

**MOLECULAR MECHANISMS OF PHENOTYPIC CHANGE IN INTACT
NOCICEPTORS**

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We have previously shown that regenerated cutaneous afferents exhibit sensitization. These changes were correlated with increased expression of neurotrophic factors in the affected skin, and receptors and ion channels in the dorsal root ganglia (DRG). Here we found that saphenous nerve injury resulted in similar alterations in gene expression in the skin innervated by the uninjured *contralateral* saphenous nerve. Several Nerve Growth Factor (NGF) and GDNF ligands were upregulated in the dorsal hindpaw hairy skin 7-21 days following contralateral saphenous nerve axotomy. This increase in expression was correlated with upregulation of several receptors and ion channels in the L2,3 DRGs, including GFR α 1, nucleotide receptors P2X3, P2Y1, and P2Y2, heat transducing channel TRPV1, and acid-sensing ion channels 1 (ASIC1) and 3 (ASIC3). Characterization of intact saphenous afferent fibers revealed that 4-5 weeks after contralateral injury, C-polymodal population had significantly reduced heat thresholds. There was also a significant increase in the proportion of mechanically insensitive-heat sensitive (CH) neurons. These findings provide evidence of peripheral mechanisms that may be involved in the development of mirror-image pain, a clinical phenomenon in which patients experience pain in the healthy region of the body contralateral to the injury site.

The changes in the response properties of intact afferents at 4-5 weeks following contralateral injury were correlated with upregulation of P2Y1 and TRPV1 in the DRGs at 28d post-injury, and we investigated the role of these two targets in the functional plasticity observed

by utilizing an *in vivo* small interfering RNA (siRNA)-mediated knockdown strategy to target the upregulation of P2Y1 and TRPV1 in the L2,3 DRGs at 28 days following contralateral injury. We found that blocking P2Y1 upregulation prevented the heat threshold reduction in C-polymodal afferents and conclude that P2Y1 plays a critical role in the heat sensitivity of these fibers. We then found that blocking the upregulation of TRPV1 blocked the recruitment of C-heat fibers. Taken together, our findings suggest that chronic exposure of intact cutaneous sensory neurons to increased endogenous levels of neurotrophic factors results in upregulation of target receptors and ion channels in the DRG and sensitization and phenotypic changes in intact sensory neurons.

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PREFACE

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1.0 INTRODUCTION

Sensory neurons are defined as those neurons that detect stimuli from either the external or internal environment and transmit this information to the CNS. Sensory input from the body and head is transmitted from the periphery by neurons located in dorsal root (DRG) and trigeminal ganglia (TG), respectively. These pseudounipolar neurons contain a peripheral process that innervates the target (skin, tongue, face, etc.) and a central process that synapses on second order neurons in the superficial dorsal horn of the spinal cord (or analogous area in the brainstem for TG neurons).

Among sensory neurons, specialized populations that can detect noxious thermal, chemical and mechanical stimuli are called nociceptors. Nociceptors can be activated by intense stimuli that are potentially damaging or damaging (noxious) to the tissues and are generally thought not to be activated by innocuous stimuli such as warming or touching, although wide-dynamic range nociceptors have been described in the viscera (Mayer and Gebhart, 1994; Traub et al., 1994) and may exist elsewhere. The consensus is that nociceptors are best distinguished by their function, and specifically by their relatively high threshold for activation, regardless of the specific stimuli to which they respond (e.g. mechanical or thermal). Among primary afferents, nociceptors are primarily small diameter unmyelinated C-fibers and lightly myelinated A δ fibers, although some A β fibers also function as nociceptors (Djouhri and Lawson, 2004). These specialized sensory neurons typically serve a protective function to prevent tissue injury when

noxious stimuli are encountered (e.g. when tissue is being burned by accidentally touching a scalding hot stove).

1.1 ANATOMY OF PRIMARY SENSORY NEURONS

Sensory neuron cell bodies are located in spinal ganglia at every vertebral level as well as in association with cranial nerves V (trigeminal), VII (facial nerve), VIII (vestibulocochlear), IX (glossopharyngeal), and X (vagus). At all vertebral levels the spinal ganglia or DRG are located on the dorsal root. Sensory neurons innervate two types of targets: somatic targets (skin, skeletal muscle and bones) and visceral targets (the inner organs). The peripheral endings of somatic nociceptors are thought to be free nerve endings, which are unencapsulated and unspecialized nerve endings. Free nerve endings are most frequently found in the skin and they penetrate the epidermis and end in the stratum granulosum.

Cutaneous sensory neurons are usually divided on the basis of the conduction velocity (CV) of their axons into C-, A δ -, and A β -fibers (Light et al., 1993). C-fibers are unmyelinated and slow conducting afferents (CV <1m/s in mouse), whereas A δ -fibers (CV<1-10 m/s mouse, <30m/s primates) are lightly myelinated, and A β -fibers (CV>10 m/s rodents, >30m/s primates) are heavily myelinated. Nociceptors are found in all classes of sensory neurons. However, as discussed above, it should be noted that in some mammals, over 50% of A-fibers can detect nociceptive stimuli (Djouhri and Lawson, 2004).

1.2 MOLECULAR PHENOTYPE OF SENSORY NEURONS

Nociceptors are an inherently heterogeneous population. They express a wide array of receptors, neurotransmitters, and other markers. In addition to being divided into three types by their CV, nociceptors are also classified as “peptidergic” and “non-peptidergic,” based on the content of peptides such as CGRP and Substance P (SP). This distinction applies primarily to C-fiber nociceptors, although peptides are also expressed in some A-fiber nociceptors. The non-peptidergic neurons are identified based on their expression of specific gangliosides that bind IB4. This distinction is useful for characterizing nociceptors because, in general, these two classes of neurons have different central and peripheral projections, growth factor dependencies, and different expression of other nociceptor-specific proteins (Snider and McMahon, 1998; Braz et al., 2005; Zylka et al., 2005; Chen et al., 2006). Non-peptidergic sensory neurons, labeled by IB4-binding, represent 41% of DRG neurons in rat (Bennett et al., 1998) and 32.5% in mice (Zwick et al., 2002), whereas 40% of DRG neurons in rat are CGRP-positive peptidergic sensory neurons (Bennett et al., 1998). During perinatal and postnatal periods, the IB4-binding non-peptidergic neurons down-regulate the trkA receptor for NGF and begin to express Ret, the canonical kinase mediating the function of GFLs (Bennett et al., 1996; Molliver et al., 1997). These two classes of nociceptors also express different nociceptive markers. In mice, transient receptor potential cation channel subfamily Vanilloid member 1 (TRPV1) is mainly expressed in IB4-negative, peptidergic neurons (Zwick et al., 2002; Woodbury et al., 2004; Lawson et al., 2008), whereas the majority of non-peptidergic neurons bind IB4 and express the ATP receptor P2X3 (Lindfors et al., 2006). Other members of the TRP channel family also expressed in sensory neurons include TRP melastatin-8 (TRPM8) and TRPA1, both of which have been proposed to play a role in cool/cold detection. TRPM8 is activated by menthol and cool stimuli

(<23-28°C), whereas TRPA1 responds to natural compounds such as mustard and cinnamon oils and responds to colder temperatures in heterologous systems (<18°C). More detail of TRP channel expression in sensory neurons will be discussed later.

1.3 SENSORY NEURON PLASTICITY

Why is it important to study the behavior of primary afferents and the molecular mechanisms that may govern their biophysical properties? While the relative contributions of peripheral vs. central mechanisms leading to sensitized pain circuits is complex, without primary afferent input, persistent pain states are rarely initiated (Cao et al., 1998; Laursen et al., 1999). Thus, in recent years many studies have examined the molecular changes in the primary afferents and their innervated tissues that might drive sensory neurons plasticity.

The signaling of target-derived growth factors such as the neurotrophin (NGF) and glial cell line-derived neurotrophic factor (GDNF) families has been implicated in this sensitization process. Previous studies have shown that constitutive overexpression of neurotrophic factors such as NGF, GDNF, Neurotrophin-3 (NT3), and artemin can alter the sensitivity of nociceptors and that this may be mediated by enhanced expression of associated ion channels and receptors in the DRGs (Stucky et al., 1999; Albers et al., 2006; Elitt et al., 2006; McIlwrath et al., 2007; Lawson et al., 2008). For example, it has been shown that overexpression of cutaneous artemin altered the levels of GFR α 3 and TRPV1 in the DRGs and cutaneous nociceptor function (Elitt et al., 2006). Similarly, overexpression of GDNF resulted in changes in the response properties of IB4-binding fibers responding to mechanical and thermal stimuli (Albers et al., 2006). Likewise, following saphenous nerve axotomy, cutaneous expression of several trophic factors, including

neurotrophins such as NGF and NT-4/5, as well as neurotrophic factors such as GDNF and Artemin, was increased in the denervated skin (Jankowski et al. 2009a).

Several studies have also provided evidence of injury-induced changes in DRG expression of several growth factor receptors and ion channels implicated in plasticity of primary afferents as well. For example, peripheral nerve injury is known to induce changes in neuropeptides such as Substance P (Bisby and Kee, 1986; Ruocco et al., 2000), GFL receptors such as GFR α 1 (Kury et al., 2001); trkA (Taniuchi et al., 1986), G-protein coupled receptors such as P2Y1 (Xiao et al., 2002), and voltage-gated ion channels (Ishikawa et al., 1999; Kim et al., 2001; Decosterd et al., 2002) in the DRGs. Axotomy also increased the total number of GFR α 3-positive neurons in the DRG (Orozco et al., 2001). The GFR α receptors for GFLs are known to be differentially regulated within sensory neurons after sciatic nerve injury. The percentage of neurons that express GFR α 2 is markedly reduced, whereas those of GFR α 1 and GFR α 3 are increased (Bennett et al., 2000). Such a phenotypic switch of injured DRG neurons may have functional implication in the generation of neuropathic pain. In recent studies, we found similar changes in several of the same ion channels and receptors in the DRGs following saphenous nerve axotomy, including GFR α 1, GFR α 3, TRPV1, TRPV2, ASIC2a, ASIC3, P2X3, P2Y1, and P2Y2 (Jankowski et al. 2009a). More recently, we showed that increased levels of artemin, GFR α 3, and TRPV1 regulate phenotypic change in cutaneous sensory neurons following nerve axotomy and regeneration (Jankowski et al., 2010, 2012). Determining whether these types of changes also occur in the DRGs of intact nerves exposed to increased neurotrophic factor levels would reveal whether the changes are growth factor-mediated or specific to injured neurons.

Inflammation is also known to evoke functional plasticity. A broad range of inflammatory mediators, including growth factors, ATP, chemokines, cytokines, and protons can sensitize sensory neurons. As a result of this sensitization, nociceptors exhibit lower thresholds and respond to innocuous stimuli (allodynia) and/or respond more robustly to noxious stimuli (Woolf and Ma, 2007). Sensitizing molecules bind to their receptors on the membrane and activate multiple intracellular signal transduction pathways in peripheral terminal that include PKC (Hucho et al., 2005), PKA (Varga et al., 2006), PI3K, MAP kinases, ERK, p38, adenylyl cyclase and JNK (Doya et al., 2005; Jin and Gereau, 2006; Mizushima et al., 2007).

Downstream of these signaling cascades are ion channels including TRP family channels (Zhang et al., 2005a; Zhang et al., 2005b; Bautista et al., 2006), voltage-gated sodium channels, G protein-coupled inwardly-rectifying potassium channels, and voltage-gated calcium channels (Kerr et al., 2001; Amaya et al., 2006). In addition to driving peripheral sensitization, inflammation can activate retrograde signals in nociceptors that increase the transcription of neuropeptides (SP and CGRP), BDNF and sodium channels, and the translation/transcription of TRP channels (Neumann et al., 1996; Mannion et al., 1999; Ji et al., 2002).

As seen in nerve injury models, inflammation has been linked with altered expression of trophic factors as well as ion channels and receptors in the DRGs. Members of the GDNF family have been shown to produce significant behavioral hyperalgesia and nociceptor sensitization when injected acutely (Malin et al., 2006). Following CFA injection, cutaneous Artemin and NGF were rapidly increased. TRPV1 expression in the DRG was found to be increased in several models of acute inflammation, including formalin-induced (Usula et al., 2010), carrageenan-induced (Zacharova et al. 2009), and CFA-induced (Molliver et al., 2005; Malin et al., 2006) inflammation. TRPV2 (Molliver et al., 2005; Shimosato et al., 2005) and several

ASICs (Voilley et al., 2001) were upregulated in the DRG in CFA-induced inflammation models as well. A number of the Gq-coupled G-protein coupled nucleotide receptors discussed earlier were also shown to be increased in the DRG after inflammation, including P2Y1, P2Y2, P2Y4, and P2Y6 (Malin et al., 2008). P2Y1 is of particular interest because it is commonly found on polymodal fibers (CPM) and colocalizes significantly with GFR α 1, the GDNF receptor (Bradbury et al., 1998; Ruan and Burnstock, 2003; Malin and Molliver, 2010). Moreover, P2Y1 has been implicated in regulating CPM thermal sensitivity following inflammation (Malin et al., 2008; Molliver et al., 2011; Jankowski et al., 2012). The literature on nerve injury and inflammation both point to the potential significance of the signaling of target-derived growth factors and the expression of receptor proteins and ion channels in the neurons that respond to them.

As discussed above, prior to this study, most work on primary afferent sensitization has been done using a variety of pathological conditions such as nerve injury or inflammation, in mice that overexpressed or were missing genes of interest, or following exogenous administration of pharmacological agents. In the experiments described here, we focus on endogenous changes in molecular expression and sensory neuron function after contralateral nerve injury. Preliminary findings revealed that many of the same neurotrophins and neurotrophic factors upregulated in the innervated hairy skin after saphenous nerve axotomy are similarly increased in the hairy skin innervated by the intact contralateral nerve. In order to better understand how endogenous upregulation of target-derived neurotrophic factors might change the properties and molecular phenotype of intact afferents, we characterized the expression of several targets in the DRG that have been implicated in nociceptor sensitization, including, but not limited to, growth factor receptors, nucleotide receptors, Acid-sensing Ion

Channels (ASICs), and TRP channels. We then recorded from these intact afferents at different timepoints to correlate any changes in their response properties with changes in expression of the genes we studied.

1.4 CLINICAL IMPLICATIONS OF MIRROR IMAGE PAIN

The results of these studies have other implications beyond simply helping us to understand how the phenotype of intact afferents might be altered after ongoing exposure to increased neurotrophic factor levels. Although neuropathic pain is perceived to arise from the tissues innervated by damaged or inflamed nerves, pathological pain can also arise from sites contralateral to (the mirror image of) the site of pathology (Maleki et al., 2000; Watkins and Maier, 2002). Indeed, the former study showed that nearly 15% of a cohort of patients with Complex Regional Pain Syndrome (CRPS) Type 1 developed pain symptoms in anatomical locations mirror opposite to where pain was first reported. Mirror image pain is known to occur in a number of other persistent pain conditions, including CRPS Type 2 (also referred to as causalgia) (Shir and Seltzer, 1991), atypical facial pain (Woda and Pionchon, 2000), idiopathic facial arthromyalgia (Woda and Pionchon, 2000), and stomatodynia (Woda and Pionchon, 2000). It is typically characterized by mechanical allodynia (Moriwaki and Yuge, 1999; Baron, 2000). That is, mirror-image pain is perceived in response to light touch/pressure stimuli (Slart et al., 1997).

How mirror-image pain develops is poorly understood. Although neuropathic pain from the area of nerve trauma can be accounted for, in part, by ectopic action potentials and

hyperexcitability (Woolf and Salter, 2000), no abnormal activity has previously been reported in the healthy contralateral nerve. Hence, abnormal contralateral peripheral nerve responsivity not believed to account for mirror image pain. A recent study showed that descending facilitation from the rostral ventral medulla (RVM) was essential for the development and maintenance of bilateral hypersensitivity following a unilateral intramuscular injection of acidic saline. It has also been proposed that mirror image pain may arise from altered spinal processing of incoming sensory information (Koltzenburg et al., 1999; Watkins and Maier, 2002). Various neurocircuits have been proposed for how altered contralateral neural processing of pain may occur (Koltzenburg et al., 1999; Ossipov et al., 2000), but whether such neurocircuits adequately account for mirror-image allodynia is unknown. Recent reports have suggested that increased DRG expression of TNF α and GFAP-positive satellite cells as well as an increase in GFAP-positive astrocytes in the dorsal horn (Hatashita et al., 2008) may have a role in the emergence of mirror image pain in neuropathic pain models, but we know very little else about the neurochemical bases of this pain phenomenon. In addition to understanding the potential impact of increased cutaneous neurotrophic factor expression on intact sensory neurons, one aim of the studies described here was to determine if there was significant primary afferent plasticity in the uninjured contralateral nerve after a unilateral injury. The model we use here might shed some light on the potential role of increased target-derived neurotrophic factor levels on primary afferent sensitization in the development of mirror image pain. Here we focus our attention on the potential molecular effectors of this primary afferent sensitization, specifically the neurotrophins and neurotrophic factors, as well as their receptors and associated ion channels in the DRG.

1.5 TROPHIC FACTORS, RECEPTOR PROTEINS, AND ION CHANNELS

1.5.1 Neurotrophins

Neurotrophins regulate survival, development, function, and plasticity of the vertebrate peripheral nervous system (Korsching, 1993; Lewin and Barde, 1996; McAllister et al., 1999; Sofroniew et al., 2001). There are four known neurotrophins in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Levi-Montalcini and Hamburger, 1951; Cohen et al., 1954; Barde et al., 1982; Phillips et al., 1990; Ibanez et al., 1993). Neurotrophins are synthesized and secreted by the targets of sympathetic and sensory neurons (Hallbook, 1999). One class of neurotrophin receptors is comprised of three members of the tropomyosin-related kinase (trk) receptor tyrosine kinase family: trkA, trkB and trkC. NGF binds trkA, BDNF and NT-4 bind trkB, NT-3 activates trkC and, to a lesser extent, trkA and trkB. P75 neurotrophin receptor (p75NTR) is a receptor that binds to all neurotrophins with a similar affinity (Rodriguez-Tebar et al., 1991). P75NTR enhances the response of trk to neurotrophins as well as increases the specificity of the Trk receptors for particular ligands. Both trkA and p75NTR receptors have nanomolar affinities for NGF and evidence from a variety of systems suggested they cooperate in transducing NGF signaling (Bibel et al., 1999; Huang and Reichardt, 2003). The expression pattern of these two receptors overlaps extensively in sensory neurons (Karchewski et al., 1999; Rifkin et al., 2000), for example, in rat dorsal root ganglion (DRG) neurons, p75NTR is expressed in almost all neurons that express trkA (Wright and Snider, 1995). *In vitro*, neurons coexpressing trkA and

p75NTR respond to lower concentrations of NGF than cells expressing trkA alone (Barker and Shooter, 1994; Hantzopoulos et al., 1994). Coexpression of both receptors increases 25-fold of the association rate of NGF with trkA (Mahadeo et al., 1994). p75NTR enhances Trk response to preferred ligands (e.g., NGF for trkA, BDNF for trkB) while attenuating responses to nonpreferred ligands (e.g., NT3 for trkA). For example, trkB is readily activated by BDNF, NT3 and NT4 in the absence of p75NTR. However, when p75NTR is coexpressed, only BDNF-induced phosphorylation of trkB remained unchanged, that induced by NT3 and NT4 was clearly reduced (Bibel et al., 1999). Other studies have shown that function-perturbing antibodies to p75NTR potentiated the NT-3 responses to trkA, suggesting that p75NTR suppresses the ability of trkA to respond to NT-3 (Clary and Reichardt, 1994).

Neurotrophins have been shown to directly bind to and dimerize trk receptors, which results in phosphorylation of tyrosine residues in the cytoplasmic domains of these receptors. Phosphorylation of these residues can activate intracellular cascades, which include Ras/extracellular signal-regulated kinase (ERK), phosphatidylinositol-3-kinase (PI-3)/Akt kinase pathway and phospholipase C-1 (PLC-1) (Huang and Reichardt, 2001). During development, peripheral targets produce a limited amount of neurotrophins that act as survival factors and are thought to match the number of surviving neurons (half of all sensory and sympathetic neurons die during ontogeny), as well as the size and innervation density of target territories (Huang and Reichardt, 2001). In addition to their role in sensory neuron development, neurotrophins are involved in neuronal regeneration and sensitization, making them ideal targets for investigation in these studies.

1.5.2 Glial Cell-line Derived Neurotrophic Factor (GDNF) Family Members

The GDNF family ligands (GFLs) consist of GDNF, neurturin, artemin, and persephin. Each of the GFLs binds a preferred GDNF-family receptor- α (GFR α) that is coupled to the plasma membrane via a glycosyl phosphatidylinositol (GPI) anchor (Airaksinen and Saarma, 2002). GDNF preferentially binds GFR α 1, neurturin preferentially binds GFR α 2, artemin preferentially binds GFR α 3, and persephin preferentially binds GFR α 4. Persephin does not act as a neurotrophic factor in the PNS (Milbrandt et al., 1998) and will not be discussed further. While GDNF and neurturin can also bind GFR α 2 and GFR α 1 (Jing et al., 1997), respectively, only artemin can activate GFR α 3 (Baloh et al., 1998b). Upon GFL binding, the GFL/GFR α complex binds to the extracellular domain of the receptor tyrosine kinase (Ret). This binding triggers autophosphorylation of the intracellular tyrosine residues which initiate a host of downstream intracellular signaling cascades. The phosphotyrosine residues can activate various signaling pathways including RAS/ERK, PI3K/AKT, p38MAPK and JNK (Takahashi, 2001), important for a variety of cell processes including survival, proliferation, and neurite outgrowth. In addition, the GPI-linked proteins cluster into lipid rafts in the plasma membrane and this location is critical for Ret signaling (Tansey et al., 2000).

1.5.2.1 GDNF

GDNF was purified from a rat glial cell line and shown to support embryonic dopaminergic midbrain neurons *in vitro* (Lin et al., 1993), creating great excitement that it might be used to treat neurodegenerative diseases, like Parkinson's Disease. In the peripheral nervous system, GFR α 1-positive sensory neurons bind the lectin IB4, are peptide poor, and some appear to be

mechanoreceptors (Silverman and Kruger, 1988; Molliver and Snider, 1997; Bennett et al., 1998; Albers et al., 2006). Mice lacking GDNF have a 23% loss of DRG neurons at postnatal day 0, fail to develop kidneys, and have deficits in the enteric nervous system (Moore et al., 1996). In addition, mice overexpressing GDNF in the skin have a 27% increase in Ret-positive, IB4-binding DRG neurons (Zwick et al., 2002), and the mechanical sensitivity of IB4-positive afferents is increased (Lawson et al., 2008). The increased mechanical sensitivity was attributed to significant increases in DRG expression of acid-sensing ion channels 2a (ASIC2a) and 2b (ASIC2b) as well as transient receptor potential (TRP) channel, subfamily A member 1 (TRPA1), which are mechanically-sensitive channels (Mullol et al., 1997; Price et al., 2000; Albers et al., 2006).

1.5.2.2 Artemin

GFR α 3, the specific GPI-linked receptor for artemin, was cloned based on the sequence homology to the other members of GFR α receptor family, GFR α 1 and α 2 (Jing et al., 1997). Artemin was cloned as a binding ligand to GFR α 3 based on the homology to GDNF and NRTN (Baloh et al., 1998). Artemin can support the survival of sensory and sympathetic neurons *in vitro* (Baloh et al., 1998) and controls the migration of sympathetic precursors *in vivo* (Nishino et al., 1999). In adult mice DRG, 20% of neurons express GFR α 3. The majority of GFR α 3-positive cells also express Ret (82%), trkA (80%), CGRP (70%), TRPV1 (99%) and peripherin

(97%), a marker of small diameter neurons that give rise to unmyelinated fibers (Orozco et al., 2001). Overexpression of artemin in skin caused a 20.5% increase in DRG neuron number and an increase in the mRNA level of GFR α 3, trkA, TRPV1, and TRPA1. Artemin overexpression also increased the behavioral sensitivity to both heat and noxious cold (Elitt et al., 2006). However, the most striking finding from this immunocytochemical characterization was that virtually all (99%) of GFR α 3-positive neurons also express the capsaicin receptor, TRPV1, suggesting that the artemin responsive population of neurons were of great importance for nociception. More recently, we showed that enhanced Artemin-GFR α 3 expression appears to be essential for TRPV1 upregulation in regenerated DRG neurons and for the recruitment of CH fibers seen after nerve axotomy (Jankowski et al., 2011).

1.5.3 Nucleotide Receptors

Previous studies have shown that increased concentrations of extracellular adenosine triphosphate (ATP) contribute to the activation of primary afferents and hyperalgesia (Burnstock, 1999). Injection of ATP into the skin elicited a foot withdrawal response in rats (Bland-Ward and Humphrey, 1997) and produced a burning pain sensation in human subjects (Hamilton et al., 2000). The activation of sensory fibers by nucleotides such as ATP, ADP, and UTP appears to occur via two families of receptors: ionotropic P2X cation receptors and metabotropic P2Y G-protein coupled receptors (GPCRs). The seven subtypes of P2X receptors have been well characterized (Chen et al., 1995; Lewis et al., 1995; Bradbury et al., 1998), and their potential role in nociception has been investigated (Cockayne et al., 2000; Souslova et al., 2000; Honore et al., 2002; Shimizu et al., 2005), but less is known about P2Y receptors. Eight members of the

P2Y family have been identified, and of these receptors, the Gq-coupled P2Y1, P2Y2, and the G_{i/o}-coupled P2Y12, P2Y13, and P2Y14 are highly expressed in rat DRGs (Ruan and Burnstock, 2003; Kobayashi et al., 2006).

1.5.3.1 P2Y1

One particular nucleotide receptor that has been hypothesized to be involved in C-polymodal (CPM) neuron heat sensitivity is P2Y1, a Gq-coupled GPCR. Several studies have reported that P2Y1 is expressed in the subpopulation of small-diameter nociceptive sensory afferents that contain P2X3, bind to the IB4, lack TRPV1, and respond to adenosine diphosphate (ADP) (Burnstock, 1999; Volonte et al., 2006; Burnstock, 2007; Cockayne et al., 2000; Malin and Molliver, 2010; Malin et al., 2008; Borvendeg et al., 2003; Kress et al., 1992; Koltzenburg et al., 1997). In mice lacking the P2Y1 receptor, CPM neurons specifically were found to have significantly elevated thresholds to heat stimuli and lower thresholds to cold stimuli compared to wild-type mice (Molliver et al., 2010). In a model of peripheral inflammation, paw withdrawal thresholds to radiant heat stimuli were reduced in P2Y1 knockout mice compared to WT mice (Malin et al., 2008). Our laboratory has also recently shown that this population of cutaneous CPM fibers undergoes a similar decrease in heat thresholds following peripheral nerve injury and regeneration and that this decrease was correlated with changes in the expression of the two purinergic receptors, P2X3 and P2Y1 (Jankowski et al., 2009). Injection of the P2Y1 agonist ADP into the hindpaw produced heat hyperalgesia in wildtype but not in P2Y1 knockout mice (Malin et al., 2010). While these studies have suggested multiple functions for the P2Y1 receptor in sensory perception, it has not been determined which neuronal cell types are

responsible for transducing P2Y1-mediated signals from the periphery. Furthermore, the impact of P2Y1 signaling on the transduction of nociceptive stimuli has not been resolved. In the present studies, we aimed to determine whether increased DRG expression of P2Y1 was correlated with functional changes in intact fibers.

1.5.4 TRP channels

Transient Receptor Potential (TRP) channels have also been targets for study in our and other labs, as their expression has been implicated in altering the functional properties of nociceptive neurons. For many years, neurons that responded to noxious thermal, chemical or mechanical stimuli (nociceptors) were identified in part by their sensitivity to the “hot” or “spicy” ingredient in chili peppers, capsaicin. Application of capsaicin to the skin leads to a psychophysical sensation of burning pain (LaMotte et al., 1991). This sensation results from excitation of nociceptive neuron terminals and their local release of inflammatory mediators (CGRP and Substance P). High doses of capsaicin in the neonatal period can ablate nociceptive neurons (Jancso et al., 1977), and repeated applications of capsaicin in the adult produce desensitization of nociceptors (Jancso et al., 1967; Jancso, 1992). This desensitization is the basis for over the counter capsaicin creams used to treat a variety of pain disorders (back pain, diabetic neuropathy, rheumatoid arthritis, etc.).

The molecular transducer in nociceptors that produces these effects upon capsaicin application was unknown until Caterina et al. cloned the capsaicin receptor (Caterina et al., 1997). They used a functional screening strategy by isolating candidate cDNA clones from DRG mRNA, injecting them into HEK293 cells and then assaying for capsaicin sensitivity using Fura-

2 calcium imaging. The newly cloned cDNA was initially named vanilloid receptor subtype 1 (VR1), based on its responsiveness to members of the vanilloid family, capsaicin and resiniferatoxin. TRPV1 contains six transmembrane domains with a short hydrophobic stretch between transmembrane regions 5 and 6 as well as three ankyrin repeat domains on the amino terminus. It is also a distant relative of *Drosophila* transient receptor potential (TRP) ion channels, which mediate depolarization of photoreceptors in the fly (Montell and Rubin, 1989). Initial characterization showed that in addition to capsaicin, TRPV1 also responds to noxious heat ($<42^{\circ}$) and acid (protons). A variety of related channels have since been cloned and are hypothesized to play important roles in thermosensation (Jordt et al., 2003; Dhaka et al., 2006). TRPV2 has been identified as an additional noxious heat channel, responding to temperatures $>52^{\circ}\text{C}$ in heterologous systems, whereas TRPV3 and TRPV4 respond to warmer temperatures (in the range of $27\text{--}39^{\circ}\text{C}$). In addition, two TRP channels that detect cold stimuli have also been identified. TRPM8 responds to innocuous cool temperatures ($\sim 8\text{--}28^{\circ}\text{C}$) and menthol whereas TRPA1 is activated by noxious cold temperatures $<17^{\circ}\text{C}$ as well as mustard oil, cinnamon oil, and bradykinin. Thus, at least in heterologous systems TRPV1, TRPV2 and TRPA1 respond to *noxious* temperatures and/or chemicals, whereas TRPV3, TRPV4 and TRPM8 can respond to non-noxious thermal stimuli.

1.5.4.1 TRPV1

TRPV1 is a nonselective cation channel gated by noxious heat ($>43^{\circ}\text{C}$), protons, and vanilloid compounds (e.g. capsaicin, resiniferatoxin) (Caterina and Julius, 2001). TRPV1 is localized to a subset of small diameter nociceptors in trigeminal and dorsal root ganglia (Caterina et al., 1997). In mouse, TRPV1 is mainly expressed in IB4-negative, peptidergic neurons (Zwick et al., 2002).

Behavioral testing revealed that TRPV1 knockout mice have normal mechanical thresholds and relatively normal thermal thresholds (Caterina et al., 2000; Davis et al., 2000). In contrast, in cultured DRG neurons from TRPV1^{-/-} animals, the electrophysiological response to noxious heat is absent (Caterina et al., 2000; Davis et al., 2000). However, in an *ex vivo* physiological preparation where the skin, DRG, and spinal cord are maintained intact in artificial CSF, the response of sensory neurons to noxious heat in TRPV1^{-/-} mice does not differ from wildtype mice (Woodbury et al., 2004). Therefore, in contrast to initial reports, TRPV1 does not appear to be necessary for the detection of noxious heat in naive animals.

However, there is considerable evidence that TRPV1 is required for inflammation-induced heat hyperalgesia. WT mice injected with carrageenan or CFA (Complete Freund's Adjuvant) become inflamed and develop robust thermal and mechanical hyperalgesia, whereas TRPV1 knockout mice do not experience thermal hyperalgesia following induction of inflammation (Caterina et al., 2000; Davis et al., 2000). Various laboratories have shown that inflammation or injury increases TRPV1 mRNA or protein (Tohda et al., 2001; Ji et al., 2002; Amaya et al., 2003; Molliver et al., 2005). In addition to increased synthesis of TRPV1, specific inflammatory mediators can sensitize TRPV1. For example pro-algesic substances released during inflammation such as ATP (Moriyama et al., 2003) and bradykinin (Chuang et al., 2001; Sugiura et al., 2002) have been shown to sensitize TRPV1 and this sensitization is absent in TRPV1^{-/-} knockouts. In addition, neurotrophic factors such as NGF are increased following inflammation (Donnerer et al., 1992) and can sensitize nociceptors by modulating TRPV1 (Chuang et al., 2001; Galoyan et al., 2003). Taken together, these studies indicate that TRPV1 is part of a common signaling pathway used by different modulators to produce hypersensitivity.

1.5.4.2 TRPA1

Using genomic based approaches, TRPA1 (previously known as ANKTM1) was cloned and characterized as a noxious cold-activated ion channel (Story et al., 2003). TRPA1 is activated at colder temperatures than TRPM8; in heterologous expression systems, TRPA1 is activated in a broader range of activation temperatures (12-27°C) with an average activation threshold of 17°C (Story et al., 2003). TRPA1 is expressed in non-myelinated C- or lightly-myelinated A-fibers lacking coexpression with neurofilament 150. TRPA1 is co-expressed with CGRP, SP and TRPV1. TRPA1 is not, however, expressed with TRPM8 (Story et al., 2003). In addition to noxious cold, TRPA1 can be activated by the pungent natural compounds present in cinnamon oil, wintergreen oil, clove oil, mustard oil and ginger (Bandell et al., 2004).

In 2006, two independent groups generated and characterized TRPA1 KO mice (Bautista et al., 2006; Kwan et al., 2006). Kwan et al. (2006) showed a substantially reduced number of mustard oil-responsive neurons using calcium imaging and reduced behavioral sensitivity to oral or injected mustard oil, whereas Bautista et al. (2006) showed the complete absence of mustard oil response behaviorally or in dissociated neurons. These groups also had conflicting results regarding the behavioral responses to noxious cold. Bautista et al. (2006) found no difference in paw lifting latency on a cold plate (0°C) and no difference in licking and flinching responses to evaporative cooling by application of acetone to the hindpaw. However, Kwan et al. (2006) found decreased behavioral responses to cold plate (0°C) and acetone in TRPA1 KO mice. For both tests, the differences between WT and KO mice were larger for females than for males. Thus, the method of measuring the behavioral responses and the gender of mice used may have contributed to the conflicting results from these two laboratories. In addition to its function in

cold nociception in sensory neurons, TRPA1 also contributes to cold hyperalgesia after inflammation and nerve injury. Following CFA injection in rats, the percent of DRG neurons expressing TRPA1 mRNA increased from 32% to 44% and 42% at 1 and 3 days post-CFA, respectively. By day 7 post-CFA, these numbers returned to normal (Obata et al., 2005). This upregulation can be induced by injection of NGF but not GDNF. Similar increases in TRPA1 mRNA were also seen after L5 spinal nerve ligation (SNL) (Obata et al., 2005; Katsura et al., 2006). Injection of an anti-NGF antibody, a p38 MAPK inhibitor or TRPA1 antisense oligodeoxynucleotide decreased the induction of TRPA1 and prevented inflammation- and nerve injury-induced cold hyperalgesia. These results provide evidence that NGF-induced TRPA1 increase in sensory neurons via p38 activation is necessary for cold hyperalgesia following inflammation or injury.

Taken together, these studies suggest that TRPA1 contributes to the transduction of mechanical, cold and chemical stimuli in sensory neurons. Moreover, since many afferents that express TRPA1 also express TRPV1 (Story et al., 2003), it is possible that interaction between these TRP channels plays a central role in the sensitization of primary afferents.

1.5.5 Acid-sensing Ion Channels (ASICs) in Primary Afferents

ASICs (acid-sensing ion channel), initially called MDEG (mammalian degenerin), BNC1 (brain Na⁺ channel 1), have been cloned in the late 90's and thereafter identified as proton-gated channels (Waldmann et al., 1996; Garcia-Anoveros et al., 1997; Waldmann et al., 1997a). In rodents, at least six ASIC subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4)

encoded by four different genes (ACCN1-4) have been described (Garcia-Anoveros et al., 1997; Waldmann et al., 1997a).

ASICs are voltage-independent, proton-gated cation channels that are permeable to Na⁺ ions (Waldmann et al., 1997a). In rodents, ASICs are widely present in the nervous system, central neurons express the ASIC1a and ASIC2 subunits (Price et al., 1996; Waldmann et al., 1996; Garcia-Anoveros et al., 1997; Lingueglia, 2007). Whereas almost all ASIC isoforms are present in primary sensory neurons of the trigeminal, vagal and dorsal root ganglia. ASIC1, ASIC2, ASIC3 are significantly expressed in the small and medium-sized nociceptive sensory neurons that are able to detect noxious thermal, chemical and high-threshold mechanical stimuli. ASIC2 and ASIC3 are also expressed in large-diameter neurons that are low-threshold mechanoreceptors (Waldmann et al., 1997b; Waldmann et al., 1997a; Liu and Simon, 2000; Bassler et al., 2001; Garcia-Anoveros et al., 2001; Voilley et al., 2001; Alvarez de la Rosa et al., 2002; Page et al., 2005; Fukuda et al., 2006). ASICs are involved in cutaneous acid-induced pain elicited by moderated pH (up to pH 6.0). For example, acid-induced cutaneous pain in healthy human volunteers can be blocked by amiloride, a non-selective ASIC channel blocker (Ugawa et al., 2002; Jones et al., 2004). The specific ASIC channel blocker A-317567 inhibits post-operative pain and inflammatory thermal hyperalgesia in rats (Dube et al., 2005). Also in rats, local peripheral application of non-selective ASIC blockers amiloride and benamil reduces cutaneous inflammatory pain (Rocha-Gonzalez et al., 2009).

Several members of ASIC family have been proposed to participate in mechanosensation. ASIC2a and ASIC3 are expressed in mechanoreceptors, including specialized cutaneous mechanosensory structures like Merkel nerve endings, Meissner corpuscles, and palisades of lanceolate nerve endings surrounding the hair shaft (Price et al., 2000; Garcia-Anoveros et al.,

2001; Price et al., 2001; Bounoutas and Chalfie, 2007). In ASIC2 knockout mice, the sensitivity of low-threshold rapidly adapting mechanoreceptors is markedly reduced (Price et al., 2000). ASIC3 knockout mice showed decreased sensitivity to noxious pinch (Price et al., 2001). Mice overexpressing cutaneous GDNF had both increased mechanical sensitivity of cutaneous C-fiber nociceptors and DRG expression of ASIC2a and ASIC2b, while those overexpressing NT-3 had increased mechanical sensitivity in myelinated afferents in correlation with increased expression of ASIC1 and ASIC3 (Lawson et al., 2008).

1.6 GOALS OF THE DISSERTATION

Although significant work has been done to study the molecular mechanisms of the functional changes in sensory neurons after injury and regeneration, less is known about the behavior of intact fibers in response to endogenous changes in expression of target-derived neurotrophic factors. Our initial finding that the hairy skin innervated by the intact saphenous nerve contralateral to a saphenous nerve injury had increased levels of several neurotrophins and neurotrophic factors motivated us to characterize those afferents to determine if they might exhibit any sensitization. It has been shown that cutaneous injection of GDNF and Artemin induced behavioral sensitization to heat in experimental animals and sensitized nociceptors *in vitro* (Malin et al., 2006), and in light of the evidence for behavioral hypersensitivity contralateral to a unilateral injury in animal models (e.g. Chacur et al., 2001; Sluka et al., 2001; Milligan et al., 2003; Radhakrishnan et al., 2003; Arguis et al., 2008) and in human subjects and

patients (e.g. Shir and Seltzer, 1991; Maleki et al., 2000; Oaklander and Brown, 2004), we hypothesized that the increased levels of neurotrophic factors might sensitize intact afferents. We first performed a detailed analysis of the time course and anatomical specificity of changes in cutaneous expression. Then, having characterized those molecular changes, we performed electrophysiological recordings using an *ex vivo* skin-saphenous nerve-DRG-spinal cord preparation to identify and characterize functional changes in the contralateral cutaneous sensory neurons that might correlate with mRNA changes. Preliminary recording experiments suggested that at 4-5 weeks following contralateral nerve injury, C-polymodal fibers had reduced heat thresholds, and there was an increase in overall prevalence of C-heat fibers not seen at 2 weeks following contralateral injury.

As discussed earlier, constitutive overexpression of neurotrophic factors can alter the sensitivity of nociceptors, and this effect may be mediated by changes in ion channel and receptor expression in the DRG (Stucky et al., 1999; Albers et al., 2006; Elitt et al., 2006; McIlwrath et al., 2007; Lawson et al., 2008). This led us to hypothesize, therefore, that the functional changes observed in intact afferents might be correlated with changes in DRG expression. In section 3.1, we discuss the mRNA expression changes in the L2,3 DRGs following contralateral nerve injury, including upregulation of many channels and receptors discussed already, such as the GDNF receptor GFR α 1, nucleotide receptors P2Y1, P2Y2, and P2X3, and TRPV1. The finding that the functional changes in intact afferents were evident at 4-5 weeks but not at 2 weeks following contralateral injury suggested that a molecular change occurring between those two timepoints might be driving this change. This focused our attention on P2Y1 and TRPV1, both of which were significantly upregulated in the DRGs at 28d but not at earlier timepoints. Using an *in vivo* siRNA-mediated knockdown strategy, we were able to

interrogate the roles of transient P2Y1 (Section 3.2) and TRPV1 (Section 3.3) upregulation in the change in response properties and molecular phenotype that was observed following contralateral saphenous nerve axotomy. Specifically, we were able to block the increased expression of the targeted genes, evaluate potential off-target effects from the siRNA injections, and then record from saphenous afferents in order to determine if and how knockdown affected the functional changes normally seen after contralateral injury.

2.0 METHODS

2.1 ANIMALS

Experiments were performed on age-matched adult (6-10 weeks old) male Swiss-Webster mice (Hilltop, Scottsdale, PA, USA) housed in the Department of Laboratory Animal Resources at the University of Pittsburgh. Mice were housed in temperature-controlled and light-controlled (12 hour light/dark cycle) room and fed standard rodent chow and water available *ad libitum*.

Animals were cared for and used in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals as well as the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2 SAPHENOUS NERVE AXOTOMY

Mice were anesthetized with inhaled isoflurane. The skin of the left hindlimb was shaved, and under aseptic conditions, a longitudinal incision was made in the skin at midthigh level, directly above the saphenous nerve, and the exposed saphenous nerve was transected using fine iridectomy scissors. The proximal and distal segments of the nerve were left adjacent to one another to allow for regeneration, and the wounds were closed using 7-0 silk suture. Animals

recovered from the anesthesia in a 28°C heated recovery chamber and were then transferred to their cages. Previously published data (Jankowski et al., 2009) from naïve, uninjured Swiss-Webster mice was used as a control. We chose to use naïve mice as controls, rather than sham-operated mice, in part because several studies have shown that in injury models such as sciatic nerve axotomy (Weissner et al., 2006) and L5 spinal nerve transection (Shortland et al., 2006), sham surgery produced a significant increase in the number of Substance P (SP)-positive neurons (Weissner et al., 2006) and an increase in ATF3-staining in the ipsilateral L4/5 DRGs (Shortland et al., 2006). The latter study also showed an increase in DRG expression of injury markers such as Galanin, Neuropeptide Y (NPY), and Nitric Oxide Synthase (NOS). A possible interpretation of these findings is that the surgical procedure to expose the nerves may have caused minor damage to axons on the nerve. Although we do not know if either of these models produced any pathology in the contralateral DRGs, given that the saphenous nerve has a superficial course near the site of axotomy, we elected not to use sham-operated mice as controls in order to avoid any incidental nerve damage from the sham surgery.

2.3 TOTAL RNA ISOLATION AND RT-PCR

Animals were anesthetized with an intramuscular injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). For each condition, one group (n=4) of these mice was used for real-time PCR analysis of changes in neurotrophic factors expression in the skin, and a separate group (n=4) of animals were used for analysis of receptor and ion channel expression in the L2-3 DRGs, at 7, 14, 21, and 28 days following contralateral saphenous nerve transection. In order to determine if there were more widespread changes in cutaneous gene expression throughout the

body, we isolated RNA not only from hairy skin innervated by the saphenous nerve but also from remote areas of skin, including the hairy skin of the thighs, back, and forelimbs. Hairy skin from the right (contralateral) hindpaw, ipsilateral and contralateral thighs, back, and ipsilateral and contralateral forelimb was rapidly removed, and RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Prior to DRG isolation, anesthetized animals were perfused transcardially with 0.9% sterile saline. RNA from DRG samples was isolated using Qiagen RNeasy Mini-Kits (Qiagen, Valencia, CA, USA). The concentration and purity of the RNA was determined by using the 260 nm absorbance recorded by a spectrophotometer (Biochrom WPA, Cambridge, UK). 1 µg of total RNA was DNase treated and then reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), and the resulting cDNA samples were stored at -80°C until needed. To determine the relative mRNA levels of the specific neurotrophic factors and receptors/ion channels of interest, cDNA were tested by real-time PCR using SYBR Green PCR Core Reagents and ABI Prism 7000 software (Applied Biosystems, Foster City, CA, USA). Forward and reverse primer sequences for NGF, Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), GDNF, neurturin, artemin, tyrosine receptor kinase B (trkB), trkC, p75, GFR α 1, GFR α 2, P2X3, P2Y1, and P2Y2 were obtained from Jankowski et al. (2009), and sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), trkA, GFR α 3, TRPV1, TRPV2, TRPV3, TRPV4, TRPA1, ASIC1, ASIC2a, ASIC3 were obtained from Elitt et al. (2006). Primer sequences are all listed in the Appendix. All genes of interest were normalized to GAPDH internal control. Change in mRNA levels was determined using the $\Delta\Delta C_t$ method, whereby the mean cycle time (C_t) of the gene of interest was subtracted from the mean GAPDH C_t value for the given sample for each of

the different conditions, and fold change was calculated as $2^{\Delta\Delta C_t}$ and a 2-fold change = 100% change.

2.4 DEVELOPMENT OF siRNAs

In order to investigate the role of specific genes in the functional changes observed in intact afferents, we utilized an *in vivo* siRNA-mediated knockdown strategy. Briefly, siRNA were designed to target the open reading frame of the gene of interest. Non-targeting controls were engineered and tested by Dharmacon (Lafayette, CO). For each gene targeted (here, P2Y1 and TRPV1), 4 different siRNAs duplexes were developed and tested as described in Jankowski et al. (2006). Briefly, mouse neuroblastoma Neuro2A cells (ATCC clone number CCL-131, Manassas, VA, USA) were maintained in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (MEMS) and 1% penicillin/streptomycin in an incubator set at 37 °C at 5% CO₂. For all experiments, cells were plated into 12-well plates at a concentration of 10,000 cells/well. Cells were grown in MEMS to 50% confluence (18–24 h) and were transfected with the siRNA of interest and TRANSIT-TKO transfection reagent in serum-free MEM to reach a final siRNA concentration of 10 nM. 24 h after siRNA transfection, the plates were harvested for RNA using the Qiagen RNeasy Mini Kit, and the siRNA duplex which produced the most significant mRNA knockdown of the target gene vs. control siRNA was selected for thiol modification. SiRNA duplexes were modified with a 5' thiol group on the sense strand and HPLC purified (Dharmacon, Lafayette, CO) to allow for conjugation with Penetratin-1, a 16 amino acid peptide corresponding to the third helix of the Antennapedia homeodomain protein which enters cells in a non-receptor-mediated manner (Derossi et al., 1994). Annealed siRNA duplexes were

resuspended in buffer provided by the manufacturer and treated with an equimolar mixture of Tris (2-carboxyethyl)phosphine at 20°C for 15 min. An equimolar ratio of Penetratin-1 (Q-Biogene, Carlsbad, CA) was added, and the mixture was heated to 65°C for 15 min and then incubated at 37°C for 1 hr before being aliquotted and stored at -80°C.

2.5 siRNA INJECTION PROTOCOL

In all animals receiving siRNA injections, a small incision was made in the skin above the right saphenous nerve (mid-thigh level), and an approximately 1 mm segment of the saphenous nerve was freed from the surrounding muscle without damaging the nerve or nearby vasculature. A chilled piece of parafilm was then placed under the freed segment of the nerve to provide stability. Penetratin-linked siRNAs were heated to 60°C for 5 minutes prior to injection, and 0.1–0.2 μ L of 80 μ M Penetratin-1 linked control, non-targeting (PenCON) or gene-targeting (PenY1 or PenV1) siRNAs were pressure injected directly into the saphenous nerve via a quartz electrode connected to a picospritzer. Injections were done at 10 psi using 7-10 pulses of 100ms duration. In order to identify an appropriate injection paradigm to block P2Y1 and TRPV1 upregulation, respectively, we measured mRNA levels in the L2,3 DRGs at 28d after contralateral saphenous nerve axotomy using two injection protocols, using 6 SW mice in each group. Both P2Y1 and TRPV1 were significantly upregulated at 28d, so we hypothesized that either a single injection of gene-targeting siRNA at 14d or a double injection done at 14d and 21d post-injury might block their respective increase in expression. For PenY1 experiments, the single injection at 14d did not block the contralateral injury-induced P2Y1 upregulation ($44 \pm 18\%$ increase vs. naïve controls), but the double injection protocol at 14d and 21d was effective

($-5 \pm 16\%$ change P2Y1 mRNA vs. controls) and was therefore used in all PenY1/PenCON experiments here. In PenV1 mice, both the single injection ($-10 \pm 8\%$ change vs. controls) and the double injection ($-16 \pm 9\%$ change vs. controls) effectively blocked the increase in TRPV1 mRNA expression. In order to minimize potential injury to the intact saphenous nerve, we chose to use a single injection of siRNAs at 14d after contralateral injury for all PenV1/PenCON experiments. It should be noted that control siRNA injections for each experimental condition were done at identical timepoints as the gene-targeting siRNA injections i.e. control siRNA injections in PenY1 experiments were performed at 14d and 21d after contralateral nerve injury, while control injection in PenV1 experiments was done at 14d after contralateral nerve injury.

2.6 PROTEIN ISOLATION AND WESTERN BLOT

Hairy skin of the hindpaw innervated by the saphenous nerve or L2-3 DRGs were isolated from naïve and injured mice, as described above, and samples were homogenized in a lysis buffer containing 10 mM Tris-HCl, 1% Sodium Dodecyl Sulfate (SDS), and protease inhibitors (1 $\mu\text{g/mL}$ pepstatin, 1 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ aprotinin, 1 mM sodium orthovanadate, and 100 $\mu\text{g/mL}$ phenylmethylsulfonyl fluoride) and then centrifuged at 4°C for 5 min in order to isolate soluble protein. Protein samples (20 μg) were boiled for 10 minutes in a denaturing buffer containing SDS and β -mercaptoethanol, separated on a 15% SDS-PAGE gel, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) overnight. Membranes were blocked and then incubated with primary antibodies overnight at 4°C (Artemin (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GDNF (1:200) (Santa Cruz), GAPDH (1:1000) (ProSci Inc., Poway, CA, USA), P2Y1 (1:800) (Alomone Labs, Israel).

Antibody binding was visualized via infrared fluorescent secondary antibodies (donkey anti-goat, donkey anti-rabbit, or donkey anti-chicken, 1:10000 concentration) on a LI-COR Odyssey system (LI-COR, Inc., Lincoln, NE, USA). Molecular weights of protein bands were estimated using a prestained standard (BioRad, Hercules, CA, USA).

2.7 IMMUNOHISTOCHEMISTRY OF WHOLE DRGS AND NEUROBIOTIN-STAINED CHARACTERIZED NEURONS

IB4-binding and TRPV1-staining in the right L3 DRGs were measured in four mice each from three groups: uninjured mice; 2 weeks post-axotomy of the left saphenous nerve; 4 weeks post-axotomy of the left saphenous nerve using the methods described previously (Christianson et al., 2006). Briefly, mice were anesthetized with a ketamine/xylazine mixture as described above and then perfused with ice-cold 0.9% saline. The right L3 DRGs were rapidly removed, fixed in 4% paraformaldehyde (PFA) for 30 minutes, and then placed in 0.1 M phosphate buffer (PB) at 4°C overnight. DRGs were embedded in 10% gelatin, fixed in 4% PFA, and then cryoprotected in 20% sucrose. The embedded ganglia were then sectioned into 60 µm slices and collected into PB. Slices were blocked and then incubated in primary for TRPV1 (1:2000, Alomone Labs, Israel), while IB4-binding was assessed using Alexa Fluor 647-conjugate (1:100, Invitrogen). Tissues were then washed and incubated in Cy3-conjugated donkey anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For each ganglion, three non-consecutive sections were chosen randomly, and then 15-21 µm stacks with 3 µm sections were imaged confocally using a 40X oil-immersion lens (Olympus, Center Valley,

PA, USA). Non-overlapping top and bottom sections within each stack were identified, and the total number of cells binding IB4 and staining for TRPV1 was measured.

2.8 PGP9.5 STAINING AND QUANTIFICATION OF INNERVATION DENSITY OF HAIRY SKIN INNERVATED BY SAPHENOUS NERVE

In order to investigate potential changes in skin innervation density, sections of the right hairy hind paw skin innervated by the saphenous nerve were isolated from mice at 14 and 28 days after contralateral saphenous nerve axotomy as well as from uninjured controls. These sections were processed immunohistochemically as described above and were then blocked and incubated in rabbit anti-PGP9.5 primary antibody (1:2000; Ultraclone, Isle of Wright, UK) overnight at room temperature. After incubation in primary antiserum, tissue was washed and incubated in Cy3-conjugated goat anti-rabbit secondary antibody (1:200; Jackson). Samples were then analyzed using confocal microscopy as described above. Nerve fiber density was then determined using Image J software as described in Anderson et al. (2010). Three non-consecutive sections of skin were used to calculate nerve fiber density. Within each image, the area to be quantified was outlined with the freehand selections tool to create a region of interest (ROI) including the epidermis and upper dermis. In order to exclude background fluorescence of collagen and other non-specific staining, the Image>Adjust>Threshold function was used. The same threshold was then applied to all images from the same region for all sections from each slide. The Analyze>Measure function was then used to measure the aggregate of the selected features. This included the total area of the ROI and the area within the ROI that was immunostained. This

was then expressed as percent area within ROI (density of staining as % of area). For each condition, 6 images each from 3 mice were analyzed and the results averaged.

2.9 EX VIVO PREPARATION AND ELECTROPHYSIOLOGICAL RECORDING

We used an *ex vivo* somatosensory preparation in which the hairy skin, peripheral nerve, DRGs, and spinal cord were dissected intact (e.g. McIlwrath et al., 2007; Lawson et al 2008) (Figure 1). Mice were anesthetized with a ketamine/xylazine mixture as described above. Mice were then perfused transcardially with ice-cold oxygenated artificial CSF (aCSF) (1.9 mM KCl, 1.2 mM KH₂PO₄, 1.3 mM MgSO₄, 2.4 mM CaCl₂, 26.0 mM NaHCO₃, and 10.0 mM D-glucose) containing 253.9 mM sucrose and no sodium ions. The right hindlimb and lumbar spinal cord were surgically removed and placed in a dish containing the circulating aCSF. The hairy hindpaw skin, saphenous nerve, L1-4 DRGs, and lumbar spinal cord were isolated intact and then transferred to a recording chamber containing oxygenated aCSF with the sodium ions replaced (127.0 mM NaCl). The skin was pinned onto a wire mesh grid with the epidermal surface of the skin up and the dermal surface of the skin perfused by the circulating aCSF. The bath temperature was gradually raised to 31°C. A suction electrode was placed on the saphenous nerve to stimulate DRG cells electrically with a square-wave pulse. A quartz recording microelectrode containing 5% Neurobiotin (NB) (Vector Labs, Burlingame, CA, USA) in 1 M potassium acetate was impaled in the DRG (either L2 or L3) for single-cell recording of action potentials. If a cell responded to the electrical stimulus, the skin was tested with a brush or von Frey filament to determine a mechanical receptive field, and if the cell had no mechanical field, the skin was tested for a response to cold (31°C) and hot (54°C) 0.9% sterile saline. In the event

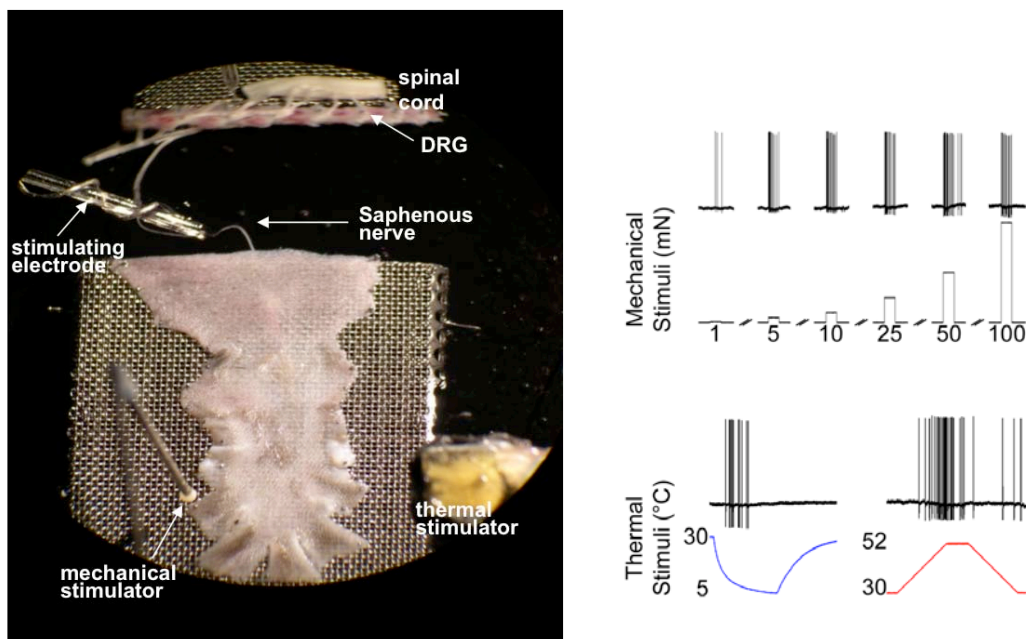


Figure 1. Diagram of ex vivo preparation. (Left panel) The hairy skin of the right hindpaw, intact, saphenous nerve, dorsal root ganglia, and spinal cord are dissected in continuity. DRG neurons are impaled with a quartz electrode, and cells of interest are identified with an electrical search stimulus. Neurons with cutaneous receptive fields have their mechanical and thermal responses characterized (stimulus paradigm on right panel). Figure adapted from Lawson et al. (2008).

there was a receptive field, a digitally-controlled mechanical stimulator (Aurora Scientific, Aurora, ON, Canada) and/or Peltier thermode (Yale University, New Haven, CT, USA) were used for characterization of the cell's response properties. The mechanical stimuli were applied for 5s at 1, 5, 10, 25, 50, and 100 mN, with 25s of break between successive stimuli to allow for recovery. A-fibers were also tested with a 100mN stimulus at 5Hz and 50Hz in order to characterize the cell's ability to adapt. The Peltier thermode applied was 3 mm² in surface area.

Data were acquired using an AxoClamp 2B (Molecular Devices, Sunnyvale, CA, USA) and Spike2 software (Cambridge Electronic Design Limited, UK), and offline analysis, including determination of conduction velocity, spike width, mechanical and thermal thresholds, and mechanical and thermal firing rates, was performed using Spike2. Cells of interest were iontophoretically injected with Neurobiotin. After completion of a recording session, ganglia with Neurobiotin-stained cells were fixed in 4% PFA for 30 minutes and then incubated in 0.1M PB. They were then embedded in gelatin, sectioned, and incubated with fluorescently-tagged Avidin-FITC (Vector Laboratories) to identify Neurobiotin-stained cells and processed for IB4-binding and TRPV1-staining as described above.

2.10 ANALYSIS OF PRIMARY AFFERENT RESPONSE PROPERTIES

Characterized fibers were first identified as A-fibers or C-fibers based on their respective conduction velocities (CV), calculated from the latency of the search stimulus and the distance from the stimulating electrode to the ganglion. Those afferents with a CV of less than 1.2 m/s were characterized as C-fibers (Kress et al., 1992; Koltzenburg et al., 1997); those with a CV between 1.2 m/s and 10 m/s were classified as A-delta ($A\delta$) –fibers, and those with CVs exceeding 10 m/s were classified as $A\beta$ -fibers. Based on their mechanical response properties and spike shape, A-fibers were classified further as low-threshold mechanoreceptors or nociceptors (Koerber et al., 1988). Mechanical thresholds were the primary determinant in this classification, but any fibers with low mechanical thresholds and an inflected spike with a broad action potential would be considered myelinated nociceptors. The low-threshold mechanoreceptors consisted of $A\beta$ rapidly adapting fibers, slowly-adapting (SA1) fibers, and $A\delta$

(D hair) hair follicle afferents, while myelinated nociceptors were classified based on their thermal sensitivities as A-mechano (AM) or A-polymodal (APM, both mechanical and thermal). C-fibers were classified by their response properties as follows: C-mechano (CM), responding only to mechanical stimuli; C-cold (CC), responding only to cooling/cold; C-mechanocold (CMC), responding to mechanical and cooling/cold stimuli; C-polymodal (CPM, including fibers responding to both mechanical and heat stimuli and those fibers responding to mechanical and both heat and cold stimuli); and C-heat (CH), responding only to heat stimuli.

2.11 STATISTICAL ANALYSIS

Mean heat and cold thresholds, RT-PCR data, and Western blot band intensities were analyzed using a one-way analysis of variance (ANOVA) with Tukey's post hoc test to determine pairwise differences between experimental groups and uninjured mice, with a p value of 0.05 set as threshold for statistical significance. Mean heat firing rates and DRG cell counts were analyzed using a two-way ANOVA with post hoc Bonferroni correction. A nonparametric, Kruskal-Wallis test with Dunn's post hoc analysis was applied to mechanical thresholds. Fisher's exact test was used to determine changes in the proportion of recorded C-fiber types in each group relative to uninjured mice. All numerical data are presented as mean \pm standard error of the mean (S.E.M.).

3.0 RESULTS

3.1 CHANGES IN MOLECULAR EXPRESSION AND SENSORY NEURON FUNCTION AFTER CONTRALATERAL INJURY

3.1.1 Unilateral saphenous nerve axotomy induces changes in expression in the contralateral skin and L2,3 DRGs

We examined changes in expression of several neurotrophin (NGF) family members, GDNF ligands, and ion channels in the skin innervated by the right saphenous nerve, at 7, 14, 21, and 28 days post-axotomy of the left saphenous nerve, collecting samples from four mice in each group. As early as seven days post-axotomy of the left saphenous nerve, we observed a statistically significant increase in artemin ($123 \pm 19\%$ increase) mRNA expression in the right (contralateral) hairy hindpaw skin, and it remained at elevated levels through the 28d timepoint. This was similar to what was seen on the ipsilateral saphenous nerve skin, where Artemin mRNA was significantly increased from 7d-21d after axotomy (Table 2, Jankowski et al., 2009). GDNF ($42 \pm 9\%$) was elevated at 21d following axotomy but were not significantly different from uninjured mice at the other timepoints (Table 1), slightly different from the ipsilateral side, where it GDNF was increased as early as 7d post-axotomy (Table 2). Neurturin ($40 \pm 15\%$) was elevated at 21d following axotomy but were not significantly different from uninjured mice at

Table 1. Changes in trophic factor levels after contralateral saphenous nerve axotomy. mRNA levels in skin after saphenous nerve axotomy, at 7, 14, 21, and 28d.

Hairy Saphenous Nerve Skin	Percent change mRNA after contralateral saphenous nerve axotomy, vs. uninjured controls			
<i>Gene Assayed</i>	7d	14d	21d	28d
NGF	9 ± 10	39 ± 20	67 ± 13	56 ± 17
BDNF	-6 ± 12	39 ± 12	50 ± 16	20 ± 8
NT3	3 ± 8	29 ± 15	46 ± 18	18 ± 10
NT4/5	82 ± 21	54 ± 13	39 ± 11	42 ± 24
GDNF	6 ± 7	30 ± 24	42 ± 9	24 ± 7
Neurturin	12 ± 8	3 ± 10	40 ± 15	-4 ± 16
Artemin	123 ± 19	40 ± 11	32 ± 5	53 ± 14
TRPV3	88 ± 18	32 ± 12	42 ± 23	20 ± 15
TRPV4	120 ± 27	120 ± 14	198 ± 17	182 ± 24

the other timepoints (Table 1), similar to the injured side where it was not increased at any timepoint (Table 2). NT-4/5 was the earliest neurotrophin with increased mRNA in the contralateral skin, evident from 7d ($82 \pm 21\%$) through 21d ($39 \pm 11\%$) after axotomy, similar to what was observed on the injured side (Table 2). Surprisingly, by 14d, BDNF was elevated ($39 \pm 12\%$), and at 21d, expression of BDNF ($50 \pm 16\%$), NGF ($67 \pm 13\%$), and NT-3 ($46 \pm 18\%$) were significantly higher than uninjured controls, changes that were not observed on the injured side. Among neurotrophin family members, only NGF was elevated at 28d ($56 \pm 17\%$). TRPV3 was upregulated ($88 \pm 18\%$) at 7d post-axotomy but returned to baseline levels from 14d-28d, whereas TRPV4 was elevated ($120 \pm 27\%$) at 7d and remained upregulated through day 28. The changes in TRPV3 and TRPV4 expression strongly correlate with what is observed on the injured side (Table 2). Generally, changes in the contralateral skin mirrored what was seen in the skin innervated by the axotomized saphenous nerve, but there were some differences,

specifically upregulation of BDNF, NT-3, and Neurturin in the contralateral skin that were not seen ipsilaterally as well as a smaller change in GDNF compared to the ipsilateral side. We also analyzed changes in protein expression of Artemin and GDNF in the hairy hindpaw skin at 14 days following contralateral saphenous nerve axotomy. We found that both GDNF ($786 \pm 192\%$) and Artemin ($180 \pm 95\%$) protein were significantly upregulated 14d after contralateral injury vs. naïve controls (Fig 2).

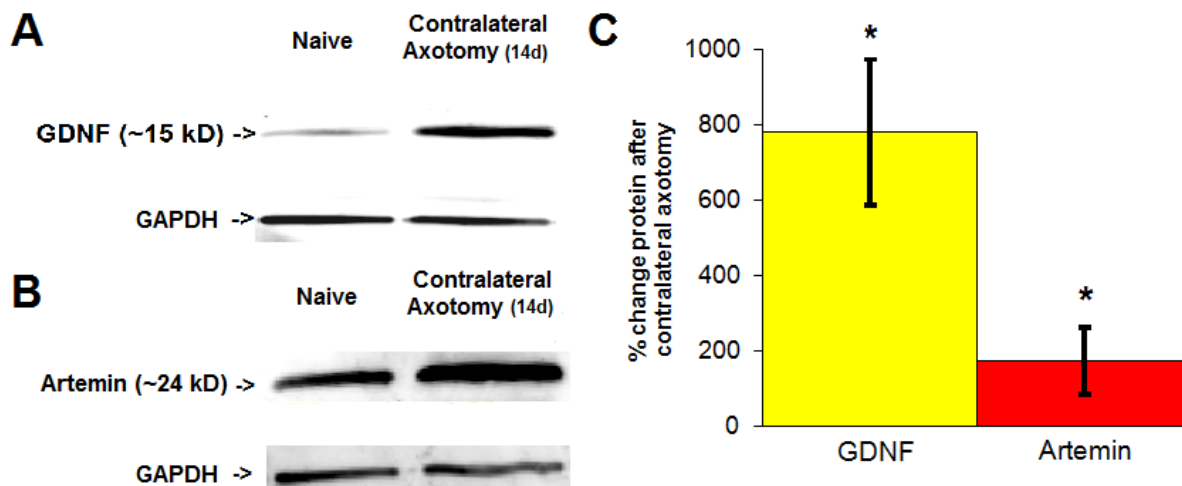


Figure 2. Artemin and GDNF Western blots. Western blot analysis of neurotrophic factor expression in the hairy hindpaw skin of mice that were uninjured and had the contralateral saphenous nerve transected 14d prior. Both GDNF (A) and artemin (B) were significantly upregulated (C) in the hairy hindpaw skin at 14 days after contralateral saphenous nerve axotomy. Significance indicated as $p < 0.05$ (*), One-way ANOVA with Tukey's post hoc test vs. uninjured mice.

Table 2. Ipsilateral changes in cutaneous trophic factor levels. Expression of several neurotrophins, neurotrophic factors, and ion channels in the hairy hindpaw skin after saphenous nerve axotomy, at 7, 14, 21, and 28d.

Hairy Saphenous Nerve Skin	Percent change mRNA after saphenous nerve axotomy (ipsi), vs. uninjured controls			
<i>Gene Assayed</i>	7d	14d	21d	28d
NGF	-8 ± 17	19 ± 15	79 ± 17	40 ± 21
BDNF	-14 ± 16	32 ± 16	47 ± 10	8 ± 15
NT3	-42 ± 22	4 ± 16	34 ± 12	19 ± 15
NT4/5	102 ± 10	38 ± 10	20 ± 10	26 ± 13
GDNF	54 ± 15	61 ± 13	50 ± 6	22 ± 15
Neurturin	-25 ± 34	-2 ± 7	14 ± 9	-11 ± 9
Artemin	131 ± 32	102 ± 7	114 ± 7	29 ± 14
TRPV3	77 ± 16	33 ± 21	8 ± 13	21 ± 18
TRPV4	140 ± 16	103 ± 18	79 ± 16	72 ± 19

In order to determine whether these changes were occurring systemically (at multiple locations), or whether they were limited to symmetrical locations, we characterized the changes in cutaneous expression bilaterally at several symmetrical sites remote from the injury. These sites included the thighs, back, and forelimbs, and were also examined at the same timepoints (7, 14, 21, and 28 days) following axotomy of the left saphenous nerve. In the hairy skin of the back and bilateral forelimbs, there was no significant change in mRNA levels of any of the genes tested at any of the four timepoints (data not shown). We did, however, detect a significant increase in expression of cutaneous artemin (61 ± 18% at 7d, 56 ± 20% at 14d, 47 ± 10% at 21d, and 77 ± 22% at 28d), GDNF(45 ± 17% at 14d and 66 ± 23% at 28d), and neurturin (40 ± 17%) in the ipsilateral thigh at various timepoints, and artemin was upregulated (42 ± 13%) in the contralateral thigh at 21 days after axotomy. Thus, the change in neurotrophic factor expression

was mostly limited to symmetrical locations in the hairy hindlimb skin including the skin innervated by the saphenous nerve and to a smaller degree in adjacent intact skin most predominantly on the side of the lesion.

Table 3. Remote changes in cutaneous trophic factor levels. Changes in expression of several trophic factors and ion channels in the skin of the thigh, back, and forelimb after unilateral saphenous nerve axotomy, at 7, 14, 21, and 28d. Significance ($p < 0.05$, One-way ANOVA with Tukey's post hoc test vs. uninjured mice) is indicated with the yellow boxes.

7d

Hairy Skin	Percent change mRNA after axotomy relative to uninjured mice				
Gene Assayed	Ipsilateral Thigh	Contralateral Thigh	Back	Ipsilateral Forelimb	Contralateral Forelimb
NGF	4 ± 12	-21 ± 14	22 ± 13	-9 ± 9	3 ± 17
BDNF	-4 ± 15	4 ± 8	11 ± 8	-8 ± 20	-1 ± 10
NT-3	-10 ± 12	-3 ± 6	2 ± 11	6 ± 14	19 ± 14
NT-4/5	-9 ± 15	4 ± 13	21 ± 16	15 ± 16	10 ± 20
GDNF	-9 ± 17	1 ± 17	5 ± 19	17 ± 10	1 ± 12
Neurturin	15 ± 14	-13 ± 11	-24 ± 15	9 ± 6	-6 ± 11
Artemin	61 ± 18	30 ± 18	8 ± 13	20 ± 15	0 ± 14

14d

Hairy Skin	Percent change mRNA after axotomy relative to uninjured mice				
Gene Assayed	Ipsilateral Thigh	Contralateral Thigh	Back	Ipsilateral Forelimb	Contralateral Forelimb
NGF	18 ± 15	3 ± 20	13 ± 10	-12 ± 17	-4 ± 15
BDNF	-10 ± 12	21 ± 22	0 ± 11	15 ± 11	10 ± 9
NT-3	12 ± 18	24 ± 11	-11 ± 18	8 ± 15	-7 ± 15
NT-4/5	15 ± 21	10 ± 16	-10 ± 11	-4 ± 12	22 ± 14
GDNF	45 ± 17	20 ± 12	1 ± 19	22 ± 15	16 ± 13
Neurturin	7 ± 8	-8 ± 16	5 ± 15	26 ± 16	32 ± 20
Artemin	56 ± 20	9 ± 11	17 ± 12	11 ± 14	-1 ± 10

21d

Hairy Skin	Percent change mRNA after axotomy relative to uninjured mice				
Gene Assayed	Ipsilateral Thigh	Contralateral Thigh	Back	Ipsilateral Forelimb	Contralateral Forelimb
NGF	12 ± 15	20 ± 11	-3 ± 13	1 ± 9	11 ± 14
BDNF	-11 ± 12	18 ± 18	-15 ± 17	15 ± 10	0 ± 10
NT-3	10 ± 12	-4 ± 6	0 ± 13	23 ± 11	18 ± 17
NT-4/5	-7 ± 21	14 ± 17	9 ± 12	22 ± 10	8 ± 19
GDNF	41 ± 22	17 ± 14	13 ± 9	-1 ± 11	-10 ± 15
Neurturin	33 ± 20	-6 ± 11	8 ± 14	19 ± 16	4 ± 12
Artemin	47 ± 10	42 ± 13	10 ± 8	23 ± 15	17 ± 13

28d

Hairy Skin	Percent change mRNA after axotomy relative to uninjured mice				
Gene Assayed	Ipsilateral Thigh	Contralateral Thigh	Back	Ipsilateral Forelimb	Contralateral Forelimb
NGF	32 ± 12	-2 ± 21	10 ± 15	-12 ± 16	24 ± 15
BDNF	16 ± 10	7 ± 14	21 ± 10	-9 ± 8	10 ± 1
NT-3	-3 ± 7	18 ± 12	5 ± 19	4 ± 9	20 ± 9
NT-4/5	0 ± 18	12 ± 15	19 ± 11	3 ± 20	-4 ± 13
GDNF	66 ± 23	28 ± 18	1 ± 16	-24 ± 10	-13 ± 13
Neurturin	40 ± 17	13 ± 15	8 ± 24	29 ± 11	-8 ± 13
Artemin	77 ± 22	14 ± 19	-5 ± 19	30 ± 15	19 ± 9

Table 4. Changes in receptor and ion channel expression in contralateral DRGs.

Expression of several neurotrophin and neurotrophic factor receptors, TRP channels, ASIC, and nucleotide receptors in the L2-3 DRGs, at 7, 14, 21, and 28d after contralateral saphenous nerve axotomy. Significance ($p < 0.05$, One-way ANOVA with Tukey's post hoc test vs. uninjured mice) is indicated with the yellow boxes.

L2-3 DRGs	Percent change mRNA after contralateral saphenous nerve axotomy, vs. uninjured controls			
<i>Gene Assayed</i>	7d	14d	21d	28d
trkA	-8 ± 9	8 ± 14	14 ± 12	17 ± 18
trkB	-12 ± 15	-25 ± 21	18 ± 16	-19 ± 17
trkC	5 ± 15	-19 ± 10	12 ± 17	-20 ± 8
p75	17 ± 9	16 ± 7	1 ± 10	-15 ± 13
GFR α 1	3 ± 8	77 ± 24	59 ± 11	100 ± 13
GFR α 2	2 ± 14	-13 ± 7	-9 ± 7	-8 ± 15
GFR α 3	4 ± 8	7 ± 20	4 ± 9	6 ± 13
TRPV1	22 ± 10	16 ± 6	1 ± 11	43 ± 15
TRPV2	37 ± 17	13 ± 9	50 ± 10	31 ± 21
TRPA1	15 ± 22	-10 ± 14	17 ± 7	-37 ± 26
ASIC1	29 ± 13	3 ± 11	44 ± 12	21 ± 15
ASIC2a	-4 ± 6	-12 ± 16	20 ± 18	-8 ± 20
ASIC3	27 ± 19	14 ± 16	47 ± 21	4 ± 15
P2X3	86 ± 22	105 ± 19	93 ± 17	66 ± 20
P2Y1	38 ± 11	44 ± 23	31 ± 16	55 ± 10
P2Y2	36 ± 9	179 ± 26	188 ± 30	49 ± 14

Next, we characterized changes in DRG expression of several neurotrophic factor receptors, as well as, a number of associated receptor/channel proteins (e.g. ASICs, TRP channels, and ionotropic P2X and metabotropic P2Y receptors) to determine if changes in neurotrophic factors seen in the dorsal hindlimb hairy skin were correlated with expression changes in the intact L2 and L3 ganglia. The GDNF receptor GFR α 1 was significantly increased at day 14 and remained elevated through day 28 (77 ± 24% at 14d, 59 ± 11% at 21d,

100 \pm 13% at 28d), but we saw no changes in expression of GFR α 2 or GFR α 3 (Table 3).

Expression of P2X3 was elevated throughout the 7d-28d period, first elevated at day 7 by 86 \pm 22%, and P2Y2 was increased at 14d (179 \pm 26%) and was still elevated at 21d (188 \pm 30%) and 28d (49 \pm 14%). At 21d only, ASIC1 (44 \pm 12%) and ASIC3 (47 \pm 21%) were significantly upregulated. By 28d, we also detect increased expression of TRPV1 (43 \pm 15%) and P2Y1 (55 \pm 10%). TRPV2 was upregulated at 7d (37 \pm 17%) and 21d (50 \pm 10%) after contralateral injury. We saw no changes in expression of any of the trk receptors, the p75 low-affinity neurotrophin receptor, ASIC2a, or TRPA1 at any of the four timepoints.

Table 5. Ipsilateral changes in receptor and ion channel expression. Expression of several neurotrophin and neurotrophic factor receptors, TRP channels, Acid-sensing ion channels (ASIC), and nucleotide receptors in the L2-3 DRGs, at 7, 14, 21, and 28d after saphenous nerve axotomy.

L2-3 DRGs	Percent change mRNA after saphenous nerve axotomy (ipsi), vs. uninjured controls			
<i>Gene Assayed</i>	7d	14d	21d	28d
trkA	23 ± 19	16 ± 27	6 ± 22	13 ± 20
trkB	-35 ± 12	-14 ± 25	0 ± 20	-20 ± 6
trkC	-5 ± 11	-10 ± 12	-9 ± 13	-30 ± 11
p75	15 ± 12	9 ± 16	1 ± 14	-5 ± 10
GFR α 1	0 ± 13	102 ± 23	159 ± 22	36 ± 17
GFR α 2	-39 ± 17	-46 ± 14	-59 ± 17	-23 ± 18
GFR α 3	41 ± 8	19 ± 9	9 ± 6	15 ± 15
TRPV1	25 ± 17	29 ± 23	8 ± 9	40 ± 8
TRPV2	34 ± 11	46 ± 16	39 ± 9	33 ± 12
TRPA1	-16 ± 17	-49 ± 14	-22 ± 14	-48 ± 19
ASIC1	11 ± 11	21 ± 13	13 ± 12	8 ± 11
ASIC2a	-35 ± 12	-13 ± 15	-7 ± 20	-14 ± 9
ASIC3	45 ± 10	35 ± 13	28 ± 13	4 ± 16
P2X3	85 ± 7	103 ± 10	49 ± 6	26 ± 30
P2Y1	13 ± 22	83 ± 20	6 ± 23	19 ± 24
P2Y2	15 ± 24	151 ± 15	255 ± 13	35 ± 28

3.1.2 Functional characterization of cutaneous afferents contralateral to nerve injury

In order to determine if there were any functional changes correlated with the increased expression seen in the skin and DRGs, we performed *ex vivo* recordings of primary afferents in the intact contralateral saphenous nerve. We characterized a total of 54 cutaneous fibers (11 A-fibers and 43 C-fibers) from 11 mice in the 2 week contralateral axotomy group and a total of 74 fibers (10 A-fibers and 64 C-fibers) from 15 mice in the 4-5 week contralateral axotomy group.

We chose the 2 week and 4-5 week timepoints because a number of genes had increased expression at 21d and 28d that were not upregulated at 7d or 14d, so we hypothesized that the increased expression might be correlated with a functional change. Additionally, there were several functional changes observed in the regenerating (ipsilateral) nerve at a similar time point (Jankowski et al., 2009). Moreover, there was significantly increased expression of GFR α 1, TRPV1, TRPV2, ASIC1, ASIC3, P2X3, P2Y1, and P2Y2 during the 3-4 week period, whereas only GFR α 1, TRPV2, P2X3, and P2Y2 were elevated at 2 weeks, suggesting that there might be measurable differences between the two timepoints. Only cells with mechanical and/or thermal cutaneous receptive fields were characterized. The two experimental groups were compared to previously published data (Jankowski et al., 2009) of fibers recorded from uninjured Swiss-Webster controls using an identical protocol to the one outlined here. We have outlined a description of the process by which the response properties of neurons with a cutaneous receptive field was characterized in Section 2.9.

3.1.3 The response properties of myelinated nociceptors do not change in the contralateral nerve at 2 weeks or 4-5 weeks post-axotomy

Among myelinated nociceptors, there was no change in the mechanical thresholds of A β -fibers (25.1 ± 5.4 mN at 2 weeks, 21.8 ± 4.7 mN at 4-5 weeks) vs. naïve controls (23.3 ± 3.7 mN) or A δ -fibers (19.4 ± 6.5 mN at 2 weeks, 18.9 ± 5.0 mN at 4-5 weeks) vs. naïve controls (20.8 ± 3.0 mN). We also did not observe any significant change in the mean firing rates or conduction velocities of either group vs. those in naïve mice (data not shown). There were not a sufficient number of fully characterized low-threshold mechanoreceptors identified in either group to

compare with naïves. We did not identify any A-fibers with heat sensitivity and therefore could not assess any change vs. uninjured controls.

3.1.4 C-polymodal fibers are sensitized to heat 4-5 weeks after contralateral injury

At 2 weeks after contralateral nerve injury the heat thresholds of CPM fibers in the intact saphenous nerve were nearly identical to control values (42.6 ± 0.9 °C) (Figure 3A). However, 4-5 weeks after contralateral injury, CPM-fibers in the intact nerve had a significantly reduced heat threshold (39.4 ± 0.9 °C) vs. uninjured controls (42.6 ± 0.5 °C), surprisingly consistent with changes in the regenerated nerve (39.8 ± 1.2 °C) reported in Jankowski et al. (2009). The reduced CPM heat threshold notably correlated with the significantly increase mRNA levels of GFR α 1 and P2Y1 (Table 2), of which the latter has been shown to be important in normal heat sensation in the CPM population (e.g. Molliver et al., 2011). Mechanical thresholds of CPM fibers were not altered in the 2 week group (16.8 ± 3.4 mN, $p=0.2$) or 4-5 week group (17.4 ± 3.1 mN, $p=0.3$) compared to uninjured mice (20.9 ± 2.2 mN), (Figure 3B). There were also no significant differences in the mean firing rates of CPM-fibers to mechanical or thermal stimulation or of CH-fibers to thermal stimulation (data not shown).

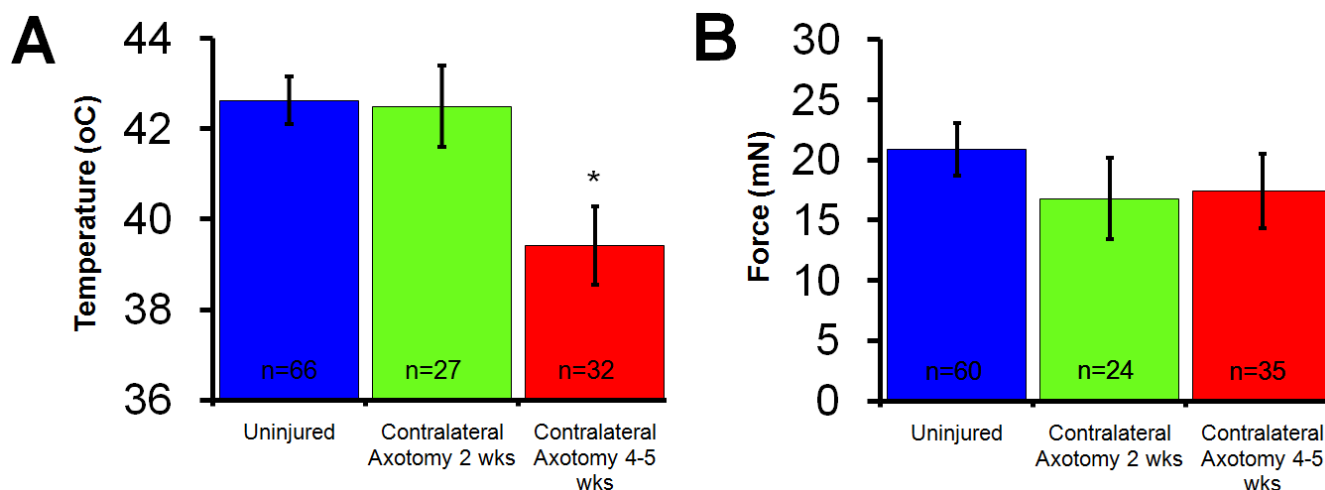


Figure 3. CPM Mechanical and Thermal Thresholds. Mean heat thresholds in characterized CPM fibers (A) were lower at 4-5 weeks following contralateral injury than at 2 weeks or in naïve mice. There was no difference in mean CPM mechanical thresholds (B) among the three groups. Significance is indicated as $p < 0.05$ (*), One-way ANOVA with Tukey's post hoc test vs. uninjured mice.

3.1.5 There is increased prevalence of C-heat fibers and decreased prevalence of C-mechano fibers 4-5 weeks after contralateral nerve injury

The heat thresholds of CH neurons in both the 2 week (43.1 ± 3.6 °C) and 4-5 week (41.5 ± 2.9 °C) groups were not statistically different from naïves (41.0 ± 1.6 °C). In addition to characterizing the response properties of individual afferents, we also examined the distribution of C-fiber types in each experimental group to determine any change in relative fiber prevalence. Although the heat thresholds and mean firing rates of CH-neurons in both experimental groups were not different from CH-fibers in naïve controls, there was a substantial increase in the proportion of CH-fibers at 4-5 weeks (20% of all characterized C-fibers vs. 4% in uninjured mice), while the 2 week contralateral group (9%) was slightly increased but not significantly different from naïve controls (Figure 4). Like the change in CPM heat threshold reduction, the increase in proportion of CH-fibers agreed with what was observed in regenerated saphenous nerves, in which 23% of cutaneous C-fibers were mechanically-insensitive CH fibers (Jankowski et al., 2009). The increase in CH prevalence here corresponded with a dramatic reduction in the prevalence of CM-fibers at the 4-5 week timepoint (2%) vs. uninjured mice (18%) that was not seen at 2 weeks (16%). Notably, this differed from regenerated nerves, in which there was a small but not significant decrease of CM prevalence to 8% following saphenous nerve axotomy (Jankowski et al., 2009). Dividing C-fibers into a heat-responsive or non-heat-responsive group revealed that the overall prevalence of heat-responsive fibers was also significantly increased in the 4-5 week contralateral group vs. controls but not in the 2 week contralateral injury mice.

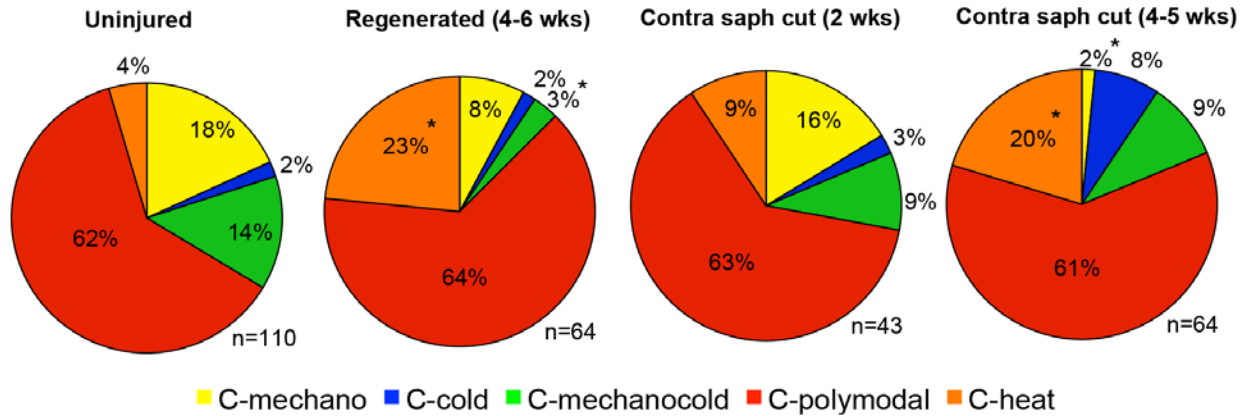


Figure 4. Recruitment of CH neurons. All characterized C-fibers were classified by their response properties as C-mechano (CM), C-cold (CC), C-mechanocold (CMC), C-polymodal (CPM), and C-heat (CH). There was a statistically significant increase in the proportion of recorded CH-fibers in the 4-5 week group vs. uninjured mice that was not seen at 2 weeks. This is similar to what is observed in regenerated fibers. Significance is indicated as $p < 0.05$ (*), Fisher's exact test vs. uninjured mice.

3.1.6 Immunostaining of functionally identified C-fibers

In the 2 week group, we intracellularly stained a total of 11 functionally characterized C-fibers from the *ex vivo* recording preparation with Neurobiotin (10 CPMs, 1 CH), and we stained 17 C-fibers in the 4-5 weeks group (15 CPM, 2 CH). Two examples of characterized stained cells are in Figure 5. In each case, the tissue was processed to assess whether the given cell bound IB4 and/or stained positively for TRPV1. In uninjured mice, nearly all CPM-fibers bind IB4 and never express TRPV1, and CH-fibers always express TRPV1 and do not bind IB4

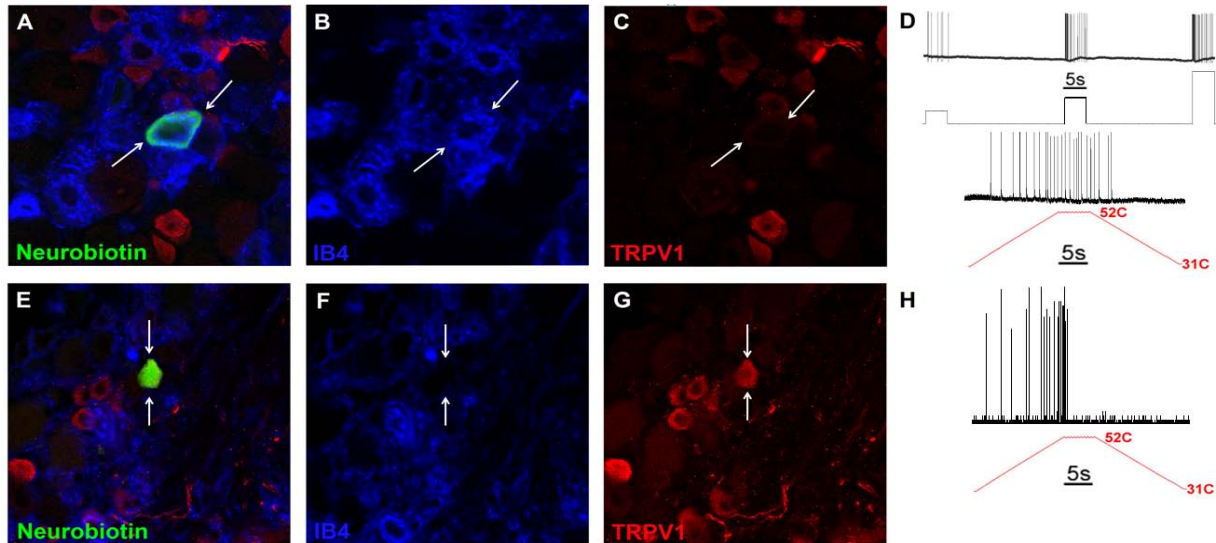


Figure 5. Immunocytochemical and electrophysiological characterization of C-polymodal (CPM) and C-heat (CH) afferents in the L2-3 DRG using the *ex vivo* preparation. Sharp electrode recordings were made from a Neurobiotin(NB)-stained CPM cell (A-D) that had characteristic mechanical and thermal responses, bound IB4, and did not express TRPV1. In contrast, a NB-stained CH neuron (E-H) responded only to heat, did not bind IB4, and stained positively for TRPV1.

(Table 6). At 2 weeks following contralateral saphenous nerve axotomy, all stained CPM fibers (10/10) bound IB4 and did not express TRPV1, while the only stained CH neuron did not bind IB4 and stained positively for TRPV1. By 4-5 weeks however, a number of CPM fibers stained positively for TRPV1 (3/15, or 20%), consistent with what is seen in the ipsilateral L2-3 DRGs (Jankowski et al., 2009). Both CH-fibers in the 4-5 week group were IB4-negative and TRPV1-positive (Table 6).

Table 6. Acquisition of mechanical sensitivity in TRPV1+ CH neurons. NB-stained CPM and CH neurons in all three groups were characterized for IB4 binding and TRPV1-staining. In only the 4-5 week contralateral group were there any CPM fibers staining positively for TRPV1. All stained CH fibers were positive for TRPV1 and negative for IB4-binding.

	Uninjured		Contra saph cut (2 wks)		Contra saph cut (4-5 wks)	
<i>Neuron Type</i>	IB4+	TRPV1+	IB4+	TRPV1+	IB4+	TRPV1+
CPM	17/26	0/18	10/10	0/10	11/15	3/15
CH	0/4	4/4	0/1	1/1	0/2	2/2

In order to determine if the increase in the prevalence of CH-fibers at 4-5 weeks and the appearance of TRPV1 polymodal fibers were correlated with any shift in the populations of IB4-binding and TRPV1-expressing neurons in the ganglion, we immunohistochemically processed L3 DRGs from a group of uninjured mice and groups of mice at 2 weeks at 4-5 weeks following a contralateral saphenous nerve axotomy. Here we found that no change in the number of TRPV1 or IB4 positive neurons after contralateral saphenous nerve section and regeneration (Figure 6).

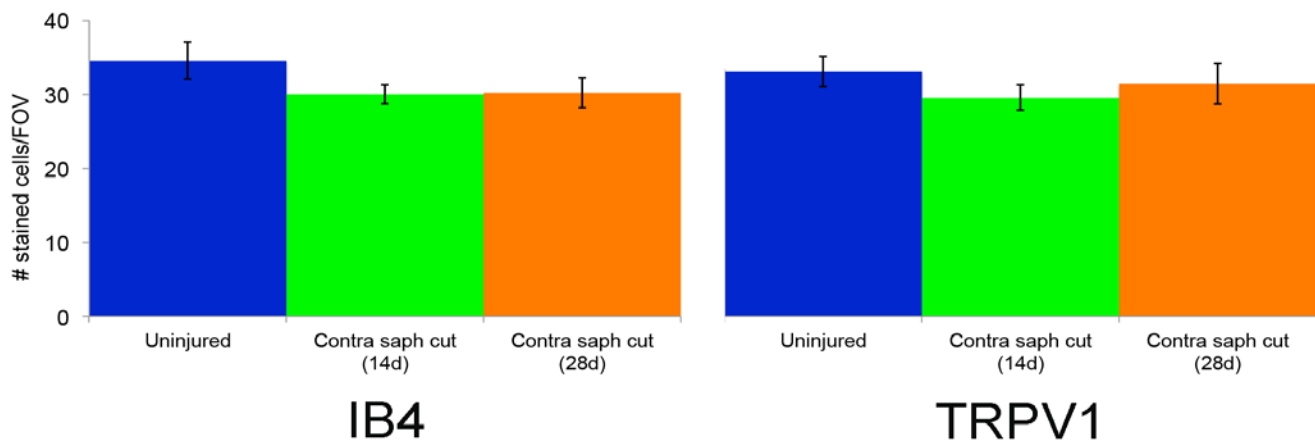


Figure 6. IB4 and TRPV1 staining in L3 DRG after contralateral injury. There is no change in total TRPV1-staining or IB4-binding in the L3 DRG at 2 weeks and 4-5 weeks following contralateral saphenous nerve axotomy.

3.1.7 Skin innervation patterns are not affected by increased expression of neurotrophic factors in the contralateral skin

In order to investigate whether the increase in neurotrophic factor expression in the skin contralateral to the nerve injury affected the cutaneous innervation, we used PGP9.5 staining to measure innervation density. We found that innervation density as measured in percentage of area staining positively for PGP9.5 was unchanged at 14 days ($2.1\% \pm 0.4\%$) or 28 days ($2.5\% \pm 0.5\%$) after contralateral saphenous nerve axotomy when compared to naïve controls ($1.9\% \pm 0.6\%$). A representative section of skin from each condition is shown in Fig. 7.

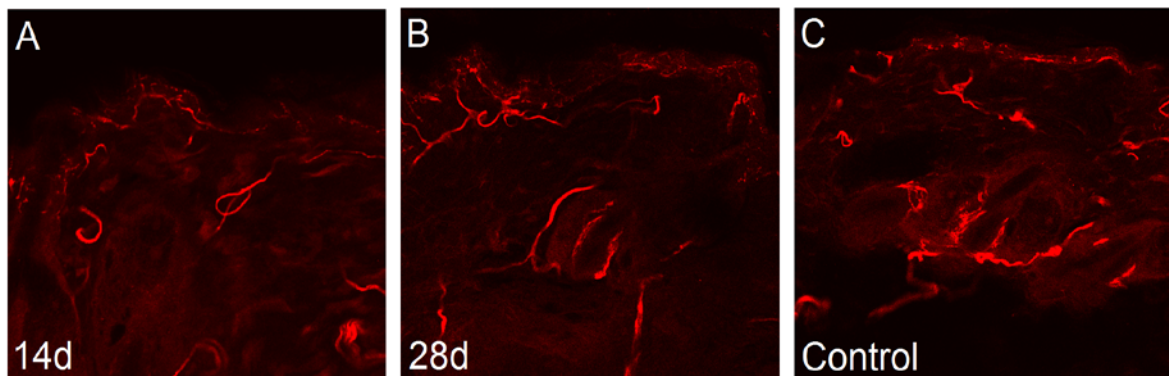


Figure 7. PGP9.5 staining of dorsal hairy hindpaw sections from control and contralateral axotomy groups. Dermal and epidermal innervations patterns at 14 days (A) and 28 days (B) following contralateral saphenous nerve axotomy are similar to those seen in uninjured mice.

3.2 THE ROLE OF INCREASED P2Y1 EXPRESSION IN THE HEAT THRESHOLD REDUCTION OF C-POLYMODAL AFFERENTS 4-5 WEEKS AFTER CONTRALATERAL INJURY

As discussed in the introduction, previous work has shown that the overexpression of neurotrophins and neurotrophic factors can alter the sensitivity of sensory neurons and that this may be mediated by enhanced expression of associated ion channels and receptors in the DRGs (Stucky et al., 1999; Albers et al., 2006; Elitt et al., 2006; McIlwrath et al., 2007; Lawson et al., 2008). Having identified here that there is enhanced cutaneous expression of GDNF in the hairy skin innervated by the saphenous nerve after contralateral injury, we focused our attention on P2Y1, which was significantly upregulated at the timepoint (28d) when we observed a heat threshold reduction in CPM fibers. P2Y1 has been shown to significantly colocalize with the GDNF-sensitive IB4-binding population (Bradbury et al., 1998; Ruan and Burnstock, 2003; Malin and Molliver, 2010), and P2Y1 has been implicated in regulating thermal sensitivity of CPM fibers following inflammation (Malin et al., 2008; Molliver et al., 2011; Jankowski et al., 2012). Here we hypothesized that increased DRG expression of P2Y1 was essential for the CPM heat threshold reduction at 4-5 weeks after contralateral injury, and we used an siRNA-mediated knockdown strategy to investigate this further.

3.2.1 *In vivo* inhibition of contralateral injury-induced P2Y1 expression

To block the contralateral injury-induced upregulation of P2Y1, we used P2Y1-targeting (PenY1) siRNAs injected into the saphenous nerve at 14d and 21d after axotomy of the contralateral saphenous nerve. Control-injected mice were injected at the same timepoints as PenY1 mice. As discussed in section 2.5, we determined that this injection paradigm produced significant knockdown of P2Y1 mRNA in a cohort of adult SW mice at 28d after contralateral saphenous nerve axotomy. We found that P2Y1 gene expression could be specifically inhibited by PenY1 injection using 6 mice in the PenY1 group and 6 PenCON controls. At 28 days following contralateral injury, P2Y1 mRNA was found to be significantly increased in the DRG after injection of control, nontargeting (PenCON) siRNA ($42\% \pm 17\%$), while injection of targeting PenY1 siRNA significantly inhibited the contralateral injury-induced increase in P2Y1 mRNA ($-5\% \pm 16\%$) (Fig. 8). Results obtained from reverse transcription PCR analysis were then verified at the protein levels (Contralateral saphenous nerve axotomy (28d) (vs. uninjured controls): $75\% \pm 12\%$; PenCON + contralateral saphenous nerve axotomy (28d): $90\% \pm 20\%$; PenY1 + contralateral saphenous nerve axotomy (28d): $10\% \pm 18\%$) (Fig. 9).

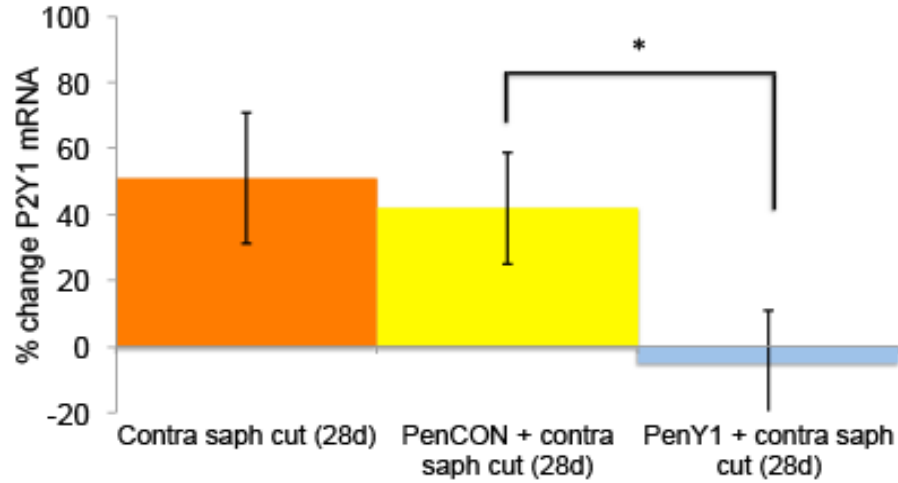


Figure 8. P2Y1 mRNA knockdown. P2Y1-targeting siRNAs injected into the intact saphenous nerve at 14d and 21d following contralateral saphenous nerve axotomy blocks increased mRNA expression of P2Y1.

In order to determine whether the inhibition of P2Y1 expression was selective, we also analyzed the expression of several additional genes following siRNA-targeted knockdown. First, we examined the expression of the ATP receptor P2X3. mRNA P2X3, which was upregulated in the L2,3 DRGs at 28 days after contralateral injury, was similarly increased following control and P2Y1 targeting siRNA injections at 28 days after contralateral injury (Contra saph cut (28d): $66\% \pm 20\%$; PenCON + contra saph cut (28d): $52\% \pm 12\%$; PenY1 + contra saph cut: $49\% \pm 20\%$). Next we analyzed the expression of TRPV1, which was significantly upregulated at 28 days following contralateral nerve injury ($49\% \pm 20\%$) (Fig 10). Following the injection of P2Y1-targeting and control siRNAs, we found that TRPV1 was significantly

increased (PenCON + contra saph cut (28d): $51\% \pm 16\%$; PenY1 + contra saph cut (28d): $47\% \pm 17\%$) (Fig. 10). We also analyzed mRNA levels of GFR α 3, whose expression was not significantly altered at 28d after contralateral injury ($13\% \pm 25\%$). Injection of P2Y1-targeting and control siRNAs revealed no change in GFR α 3 mRNA levels (PenCON + contra saph cut (28d): $15\% \pm 22\%$; PenY1 + contra saph cut (28d): $3\% \pm 21\%$) (data not shown).

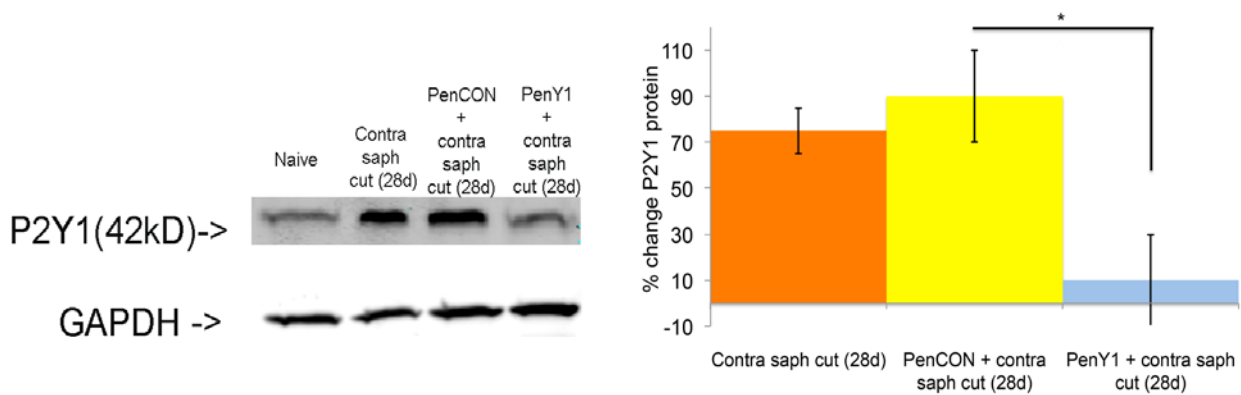


Figure 9. P2Y1 protein knockdown. Western blot analysis of pooled L2,3 DRG samples showed that PenY1 is able to block the contralateral injury-induced upregulation of P2Y1 protein.

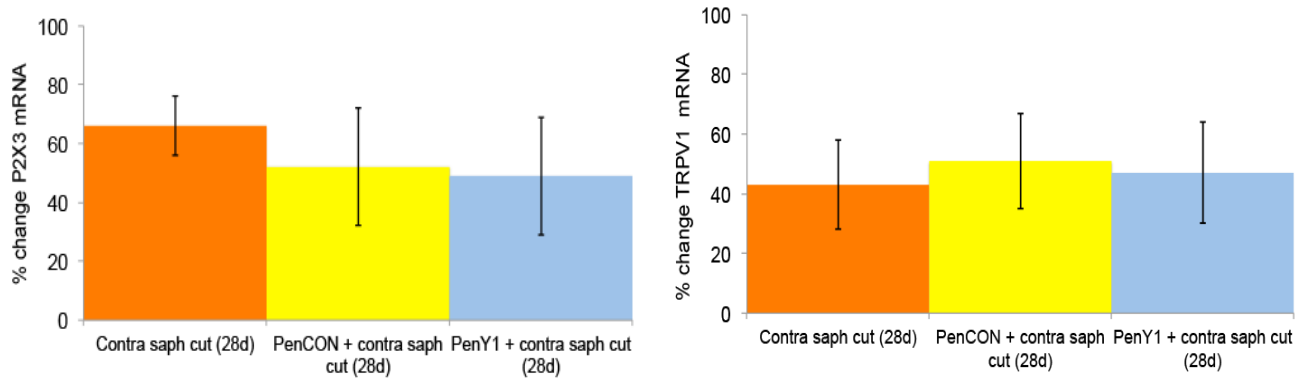


Figure 10. Off-target effects of PenY1 injection. Messenger RNA levels of P2X3 and TRPV1 are unaffected by PenY1 injection. These results suggest that off-target effects of the P2Y1-targeting siRNAs are minimal.

3.2.2 Characterization of primary afferents in the intact saphenous nerve following P2Y1 knockdown at 4-5 weeks after contralateral saphenous nerve axotomy

A total of 147 primary cutaneous neurons were intracellularly recorded and physiologically characterized from age-matched adult Swiss-Webster mice (n=28) from 28 days to 35 days following axotomy of the left (contralateral) saphenous nerve. Of these 28 mice, 15 were injected with P2Y1-targeting siRNAs at 14d and 21d after contralateral injury, and 13 were injected with control siRNAs. The results from these two experimental groups were compared with each other, to the contralateral injury group discussed in section 3.1, and to previously published results from naïve Swiss-Webster mice (Jankowski et al., 2009a).

3.2.3 P2Y1 knockdown blocks the contralateral injury-induced decreased in CPM heat thresholds

Mice injected with control siRNAs into the intact saphenous nerve had CPM heat thresholds which were significantly reduced ($38.2^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$) ($P = 0.01$) compared to previous naïve mouse data ($42.6^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) (Fig. 11). However, inhibition of P2Y1 with P2Y1-targeting siRNA injections resulted in significantly higher thermal thresholds ($41.7^{\circ}\text{C} \pm 0.9^{\circ}\text{C}$; $P < 0.05$ vs. PenCON mice, $p=0.3$ vs naïve mice) (Fig. 11). Additionally, heat thresholds of CPM neurons from mice that received P2Y1 siRNA at this time point were not statistically different from naïve CPMs, ($p=0.3$ vs naïve mice). In agreement with data from Section 3.1, CPM mean firing rates were not changed in either PenCON or PenY1 mice. We also determined the peak instantaneous firing rate of CPM neurons to heat and found no differences in firing rate in either experimental group. Although we previously showed that P2Y1 knockdown reduced cold thresholds in CPM fibers responding to cold (Jankowski et al., 2011), we did not observe any change in CPM cold thresholds in PenCON or PenY1 mice (Naïve: $16.6^{\circ}\text{C} \pm 0.6^{\circ}\text{C}$; PenCON + contra saph cut (4-5 wks): $17.7^{\circ}\text{C} \pm 1.8^{\circ}\text{C}$; PenY1 + contra saph cut (4-5 wks): $15.0^{\circ}\text{C} \pm 1.4^{\circ}\text{C}$), though PenY1 mice approached significance vs. naïves ($p=0.1$). CPM mechanical thresholds were not altered in either PenCON or PenY1 mice (Naïve: $21.2 \text{ mN} \pm 2.2 \text{ mN}$; PenCON + contra saph cut (4-5 wks): $19.4 \text{ mN} \pm 4.1 \text{ mN}$; PenY1 + contra saph cut (4-5 wks): $17.4 \text{ mN} \pm 4.2 \text{ mN}$).

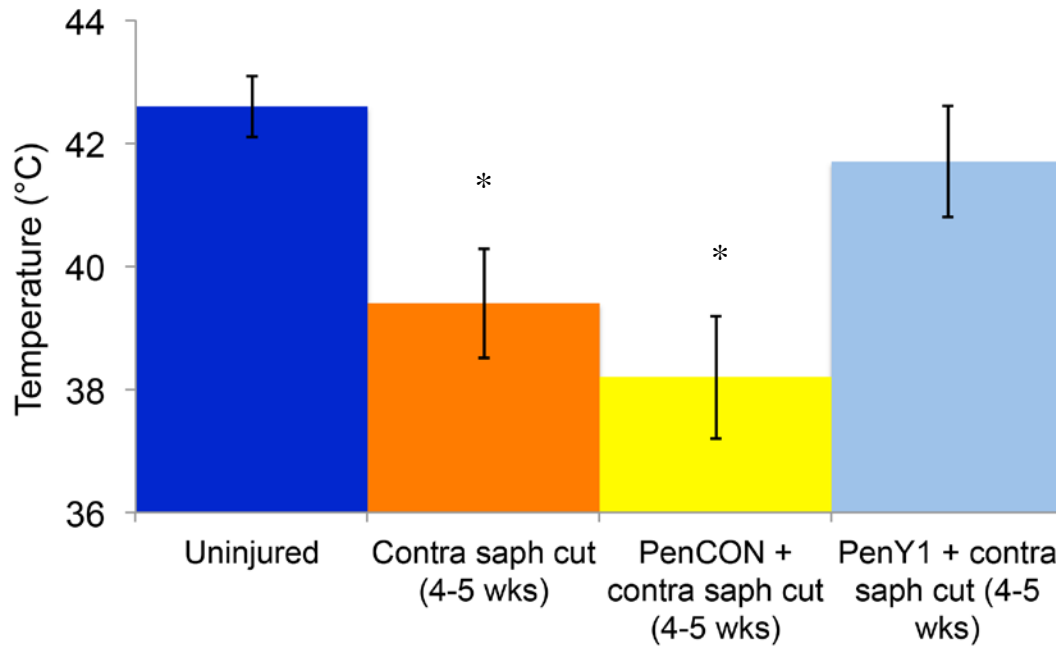


Figure 11. CPM heat thresholds after PenY1 injection: In PenY1-injected mice, mean heat thresholds in characterized CPM fibers were significantly higher than in control-injected mice and approached heat thresholds seen in CPM fibers from naïve mice. * $p < 0.05$ vs. Uninjured mice, One-way ANOVA, Tukey's post hoc test.

3.2.4 P2Y1 knockdown does not affect recruitment of CH neurons induced by contralateral injury

The heat thresholds of TRPV1-immunopositive, IB4-negative CH fibers in both the PenCON (39.9 ± 3.3 °C) and PenY1 (40.2 ± 4.1 °C) groups were not statistically different from naïves (41.0 ± 1.6 °C). In addition, both control-injected and PenY1-injected mice had significantly increased overall CH prevalence at 4-5 weeks following contralateral injury (PenCON + contra saph cut (4-5 wks): 26%, $p < 0.05$, Fisher's Exact Test; PenY1 + contra saph cut (4-5 wks: 19%, $p < 0.05$, Fisher's Exact Test) when compared to naïve mice (4% CH prevalence) (Fig. 12). A similar significant increase in CH fiber prevalence was also found in regenerated CH fibers after saphenous nerve axotomy (Jankowski et al., 2009). Thus, the injection of PenCON and PenY1 siRNAs had no effect on the normal increase in CH prevalence of cutaneous fibers following contralateral nerve injury. The decrease in overall C-mechano prevalence found in the 4-5 week contralateral injury group (2% prevalence vs. 18% in naïve controls) was not evident in either injection group, surprisingly, as CM prevalence was 8% for PenCON mice and 9% for PenY1 mice. Statistically, however, the CM prevalence in the injected groups was neither different from naïves ($p = 0.3$ for PenCON, $p = 0.4$ for PenY1) or the 4-5 week contralateral axotomy group ($p = 0.2$ for PenCON, $p = 0.3$ for PenY1).

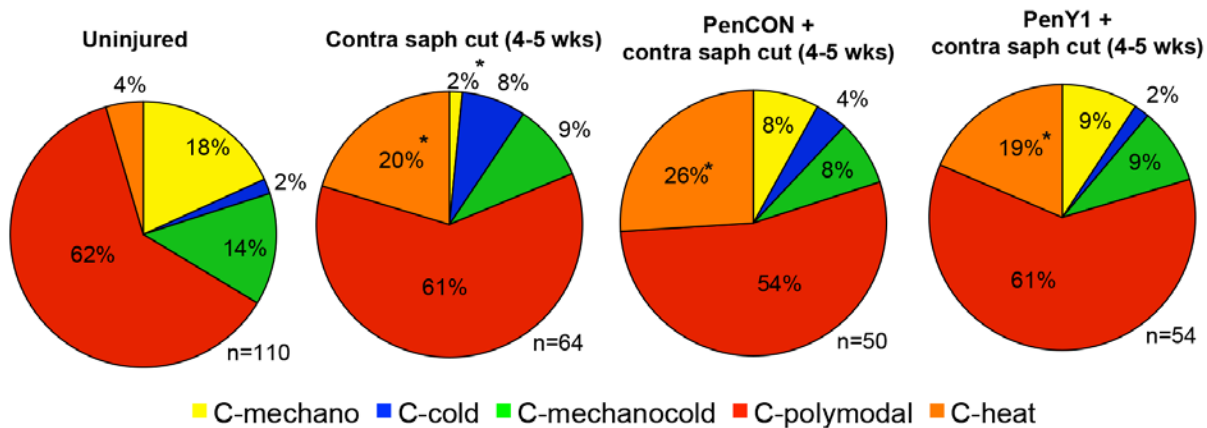


Figure 12. CH recruitment after PenY1 injection. Recruitment of CH neurons at 4-5 weeks after contralateral injury is not affected by knockdown of P2Y1 expression. There was still a statistically significant increase in the proportion of recorded CH-fibers in both PenCON and PenY1 mice vs. uninjured mice. Significance is indicated as $p < 0.05$ (*), Fisher's exact test vs. uninjured mice.

3.2.5 Immunohistochemical characterization of CPM and CH neurons following and P2Y1 knockdown at 4-5 weeks after contralateral saphenous nerve axotomy

A total of 16 cutaneous C fibers were intracellularly labeled, recovered for processing, and immunohistochemically characterized in the injected mice. Among control siRNA injected mice, nearly all (6/7, 86%) of stained CPM fibers bound IB4, while one CPM fiber stained mice, nearly all (6/7, 86%) while one CPM fiber stained positively for TRPV1 (14%) (Table 5). The presence of TRPV1+ CPM fibers in both groups follows what is seen in the 4-5 week contralateral injury mice (from section 3.1) and in regenerated fibers (Jankowski et al., 2009).

Table 7. NB-stained characterized C-fibers in PenY1 and PenCON mice. NB-stained CPM fibers staining positively for TRPV1 are evident in both PenCON and PenY1 mice at 4-5 weeks following contralateral saphenous nerve axotomy. All stained CH fibers were negative for IB4, and all but one was TRPV1-immunopositive.

	Uninjured		Contra saph cut (4-5 wks)		PenCON + contra saph cut (4-5 wks)		PenY1 + contra saph cut (4-5 wks)	
<i>Neuron Type</i>	IB4+	TRPV1+	IB4+	TRPV1+	IB4+	TRPV1+	IB4+	TRPV1+
CPM	17/26	0/18	11/15	3/15	6/7	1/7	3/4	1/4
CH	0/4	4/4	0/2	2/2	0/3	2/3	0/2	2/2

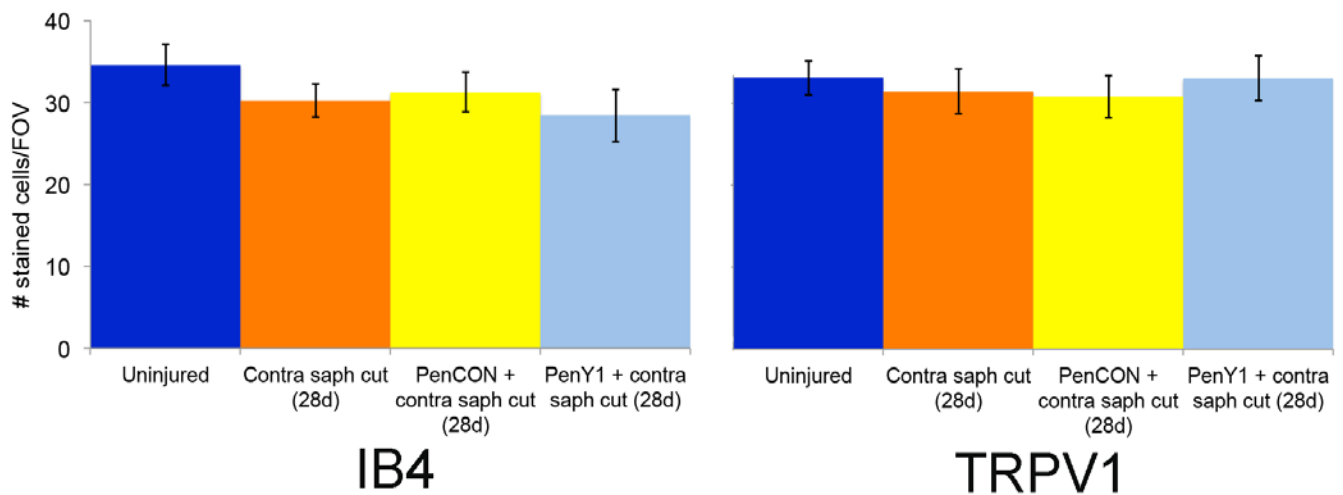


Figure 13. IB4 and TRPV1 staining in PenY1 mice and controls. PenCON and PenY1 mice show no change in total TRPV1-staining or IB4-binding in the L3 DRG at 4-5 weeks following contralateral saphenous nerve axotomy.

Two out of three (67%) stained CH fibers in the PenCON group were positive for TRPV1, and none bound IB4. In PenY1 mice, 3/4 (75%) CPM fibers bound IB4, and 1/4 (25%) was TRPV1+. Of the two stained CH fibers in the PenY1 group, both were positive for TRPV1. Although the total number of characterized stained cells in these groups was fairly small, the data generally agree with data from mice only receiving a contralateral injury, suggesting that P2Y1 upregulation is not essential for the observed shift in phenotype. We looked at whole DRG staining for IB4 and TRPV1 in the injected groups to determine if there was any shift in the total number of neurons in each population. Our analysis revealed that the appearance of TRPV1+ CPM fibers is apparently not due to *de novo* TRPV1 expression, as there was no difference in the numbers of TRPV1-containing DRG neurons in either group when compared to naïve controls and the contralateral injury (28d) group (Naïve: 33.1 ± 2.1 ; PenCON + contra saph cut (28d): 30.8 ± 2.6 ; PenY1 + contra saph cut (28d): 33.0 ± 2.7) (Fig. 13). Total IB4 binding was also unaffected under any condition (Naïve: 34.5 ± 2.5 ; PenCON + contra saph cut (28d): 31.2 ± 2.4 ; PenY1 + contra saph cut (28d): 28.4 ± 3.2). (Fig. 13).

3.3 THE ROLE OF INCREASED TRPV1 EXPRESSION IN THE RECRUITMENT OF C-HEAT FIBERS AT 4-5 WEEKS AFTER CONTRALATERAL INJURY

Having shown that enhanced P2Y1 expression was essential for the CPM heat threshold reduction in intact saphenous afferents after contralateral nerve injury, we hypothesized that TRPV1 upregulation had a critical role in the increase in overall CH prevalence at the 4-5 week timepoint after contralateral injury. We showed that cutaneous Artemin was significantly increased in message and in protein by 14d, and nearly 99% of cells expressing the Artemin receptor GFRa3 also express TRPV1 (Orozco et al., 2001; Elitt et al., 2006), which was significantly upregulated in the contralateral L2,3 DRGs at 28d after contralateral injury. Moreover, nearly all CH neurons in mice express TRPV1 (Lawson et al., 2008). In the following experiments, we targeted TRPV1 upregulation using TRPV1-targeting siRNAs in order to investigate whether increased TRPV1 expression is required for the CH recruitment we observed.

3.3.1 Confirmation of TRPV1 mRNA knockdown with TRPV1-targeting siRNA

In order to investigate the role of increased TRPV1 expression in the functional changes observed at 4-5 weeks after contralateral saphenous nerve axotomy, we used TRPV1-targeting siRNAs injected into the intact saphenous nerve to confirm that we could effectively block the TRPV1 mRNA upregulation. At 28 days following contralateral injury, TRPV1 mRNA was found to be significantly increased in the DRG after injection of control, nontargeting (PenCON) siRNA ($47\% \pm 8\%$; Fig. 14). Injection of targeting PenV1 siRNA significantly inhibited the contralateral injury-induced increase in TRPV1 mRNA ($-10\% \pm 8\%$) (Fig. 14). We attempted

several TRPV1 Western blot assays of L2,3 DRG protein with several different protein isolation techniques and antibodies but did not identify a protocol that produced reliable, reproducible data.

We analyzed mRNA levels of a number of other genes to determine the selectivity of TRPV1 knockdown. We performed RT-PCR analysis of P2Y1, primarily expressed on nonpeptidergic afferents, and P2Y2, which is highly expressed in TRPV1+ neurons. P2Y1, which was upregulated in the L2,3 DRGs at 28 days after contralateral injury, was similarly increased following control and TRPV1 targeting siRNA injections at 28 days after contralateral injury (Contra saph cut (28d): $55\% \pm 10\%$; PenCON + contra saph cut (28d): $53\% \pm 5\%$; PenV1 + contra saph cut: $44\% \pm 8\%$) (Fig 14). Next we analyzed the expression of P2Y2, which was significantly upregulated at 28 days following contralateral nerve injury ($55\% \pm 18\%$) (Fig. 14) and which has been shown to be critical for thermal nociception and function of TRPV1 (Malin et al., 2008).

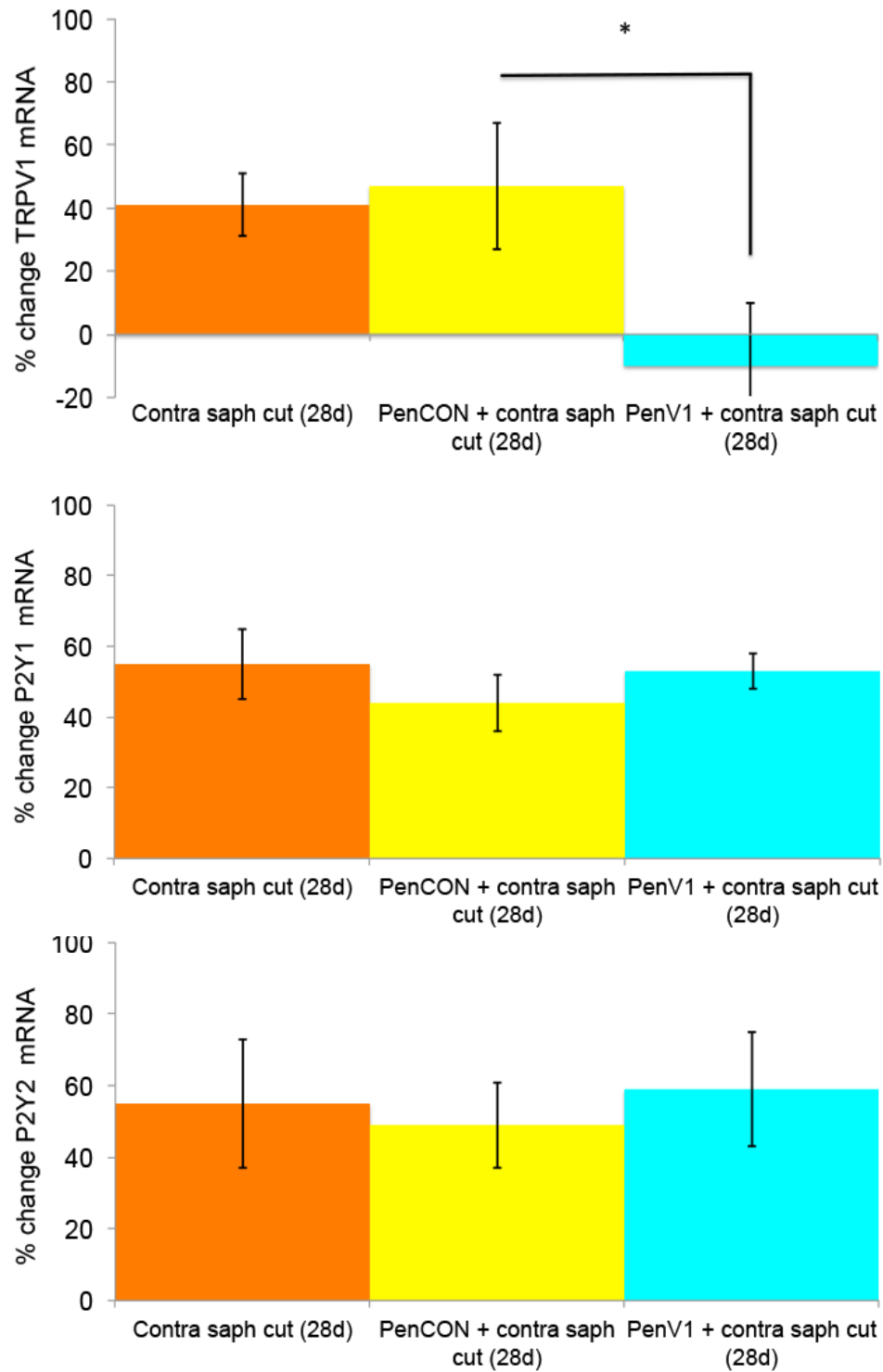


Figure 14. Effect of PenV1 on TRPV1, P2Y1, and P2Y2 mRNA. TRPV1-targeting siRNA (PenV1) can block the contralateral injury-induced upregulation of TRPV1 but does not affect P2Y1 and P2Y2 upregulation. The results suggest that off-target effects of PenV1 are minimal. * $p < 0.05$ vs. PenCON + contra saph cut.

Following the injection of TRPV1-targeting and control siRNAs, we found that P2Y2 was still significantly increased (PenCON + contra saph cut (28d): $59\% \pm 18\%$; PenV1 + contra saph cut (28d): $49\% \pm 12\%$) (Fig. 14). As in the previous section, we also analyzed mRNA levels of GFR α 3, whose expression was not significantly altered at 28d after contralateral injury ($13\% \pm 25\%$) and whose increased expression was shown to be critical for CH recruitment after nerve injury (Jankowski et al., 2010). Injection of TRPV1-targeting and control siRNAs revealed no change in GFR α 3 mRNA levels (PenCON + contra saph cut (28d): $15\% \pm 7\%$; PenV1 + contra saph cut (28d): $-6\% \pm 9\%$) (data not shown).

3.3.2 Characterization of primary afferents after TRPV1 knockdown at 4-5 weeks following contralateral saphenous nerve axotomy

A total of 116 primary cutaneous neurons were intracellularly recorded and physiologically characterized from 29 nerve injured Swiss-Webster mice (PenCON: n=13; PenV1: n=16). Of the characterized cells, 53 cells came from mice that received nerve cut of the left saphenous nerve plus control (PenCON) siRNA injection into the right saphenous nerve 14d post-injury. The remaining 63 cells came from mice that received nerve cut of the left saphenous nerve plus TRPV1-targeting (PenV1) siRNA injection into the right saphenous nerve 14d post-injury. The results from these experimental groups were compared with one another and to the mice described in Section 3.1 (contralateral injury only), as well as previously published results from naive SW mice (Jankowski et al., 2009a).

3.3.3 TRPV1 knockdown does not affect the CPM heat threshold reduction normally seen at 4-5 weeks after contralateral saphenous nerve axotomy

In PenCON and PenV1 mice, CPM heat thresholds were significantly reduced (PenCON + contra saph cut (4-5 wks): $38.9 \pm 0.9^{\circ}\text{C}$, $p < 0.01$; PenV1 + contra saph cut (4-5 wks): $39.4 \pm 0.8^{\circ}\text{C}$ ($p < 0.01$) compared to previous naïve mouse data ($42.6 \pm 0.5^{\circ}\text{C}$) (Fig 15). CPM mean firing rates to heat were not changed in either PenCON or PenY1 mice. We also determined the peak instantaneous firing rate of CPM neurons to a heat ramp and found no differences in firing rate in either experimental group. As seen in the PenY1/PenCON mice, we did not observe any change in CPM cold thresholds in PenCON or PenY1 mice (Naïve: $16.6^{\circ}\text{C} \pm 0.6^{\circ}\text{C}$; PenCON + contra saph cut (4-5 wks): $17.5^{\circ}\text{C} \pm 2.7^{\circ}\text{C}$; PenV1 + contra saph cut (4-5 wks): $16.4^{\circ}\text{C} \pm 1.9^{\circ}\text{C}$). CPM mechanical thresholds were not altered in either PenCON or PenV1 mice (Naïve: $21.2 \text{ mN} \pm 2.2 \text{ mN}$; PenCON + contra saph cut (4-5 wks): $16.9 \text{ mN} \pm 4.7 \text{ mN}$; PenV1 + contra saph cut (4-5 wks): $19.1 \text{ mN} \pm 3.2 \text{ mN}$). In summary, TRPV1-targeting siRNA injections had no apparent effect on the response properties of CPM fibers after contralateral injury.

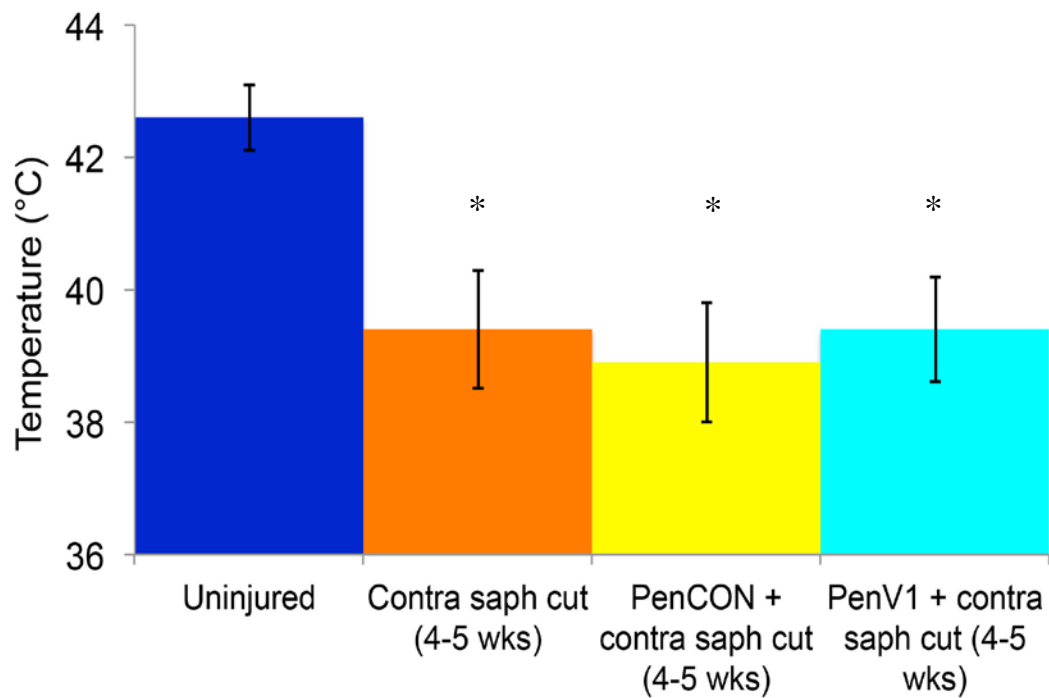


Figure 15. CPM heat thresholds in PenV1 mice and controls. Knockdown of TRPV1 expression with PenV1 does not affect the CPM heat threshold reduction normally seen at 4-5 weeks after contralateral injury. *($p < 0.05$ vs uninjured controls, One-way ANOVA, Tukey's post hoc test).

3.3.4 TRPV1 knockdown blocks the recruitment of newly functional CH fibers at 4-5 weeks following contralateral injury

As in the PenY1/PenCON experiments, the heat thresholds of CH fibers in both the PenCON (41.9 ± 3.0 °C) and PenV1 (41.0 ± 3.6 °C) groups were not statistically different from naïves (41.0 ± 1.6 °C). CH fibers here also showed no significant changes in thermal threshold or firing rate in either condition. However, the prevalence of CH fibers significantly decreased significantly after PenV1 injection (9%) vs. 21% seen in PenCON mice (Fig. 16) and 20% seen in the 4-5 week contralateral injury group (Fig. 16). The difference in CH prevalence between PenCON and PenV1 mice is statistically significant ($p=0.04$, Fisher's Exact Test). Surprisingly,

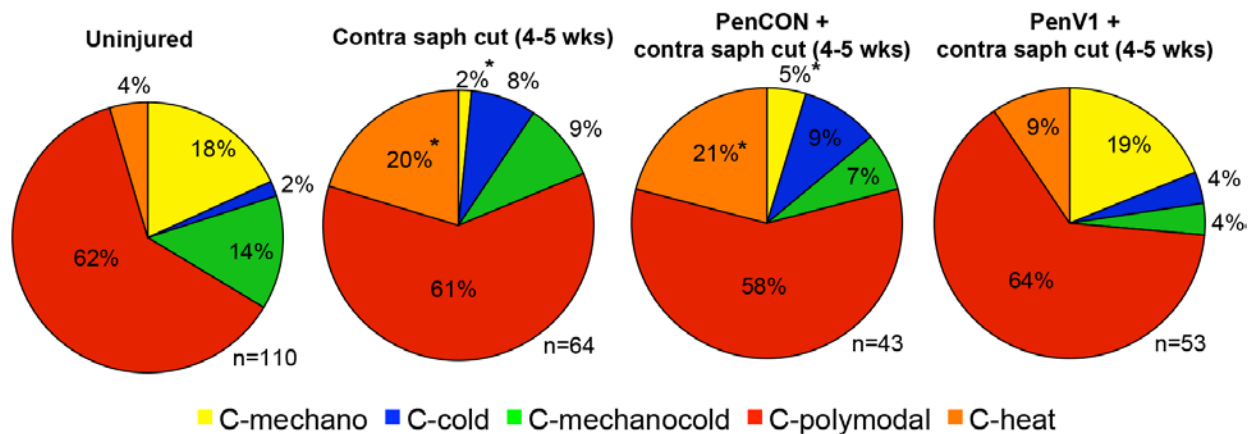


Figure 16. PenV1 injection blocks CH recruitment. Knockdown of TRPV1 expression with PenV1 almost completely blocks CH neuron recruitment seen at 4-5 weeks after contralateral injury. There was still a statistically significant increase in the proportion of recorded CH-fibers in PenCON mice vs. uninjured mice. * $p<0.05$, Fisher's exact test vs. uninjured mice. PenV1 mice also had an increase in CM prevalence to a level normally seen in uninjured mice.

we also saw that PenV1 injection reversed the reduction in overall C-mechano prevalence to levels seen in uninjured mice (19% in PenV1 mice vs. 18% in naïve controls) (Fig 16). In summary, PenV1 injection blocked CH neuron recruitment and the reduction in CM prevalence after contralateral nerve injury.

3.3.5 Neurochemical identity of CPM and CH neurons after TRPV1 knockdown at 4-5 weeks following contralateral saphenous nerve axotomy

A total of 16 cutaneous C-fibers were intracellularly labeled, recovered, and immunohistochemically characterized in nerve-injured mice from the PenCON and PenV1 groups. In PenCON mice, the only stained CH fiber was TRPV1+ and did not bind IB4 (Table 5). In CPM fibers in PenCON mice, 2/3 were IB4+ and 1/3 (33%) was TRPV1+, similar to what was observed in the 4-5 week contralateral injury group (Table 8). In PenV1 mice, 4/4 CH fibers stained positively for TRPV1 and did not bind IB4 (Table 8). 6/8 stained CPM fibers in the PenV1 group were IB4+, TRPV1-, but one fiber was IB4-, TRPV1+. The overall number of stained cells here is too few to find any significant difference relative to uninjured mice, but it is still an important finding that we see TRPV1+ CPM fibers in both experimental groups, since we see them at 4-5 weeks following contralateral injury and have not observed any in naïve mice. These results suggest that TRPV1 upregulation is not essential for the apparent shift in phenotype of these TRPV1+ cells.

Since TRPV1-targeting siRNA effectively blocked the recruitment of CH neurons at 4-5 weeks following contralateral injury, we sought to determine if total TRPV1 staining in the DRG was affected by PenV1 injection. Although we saw no change in total TRPV1 or IB4 staining

after contralateral injury alone, we wanted to determine if PenV1 was able to alter the total number of neurons in the TRPV1+ and IB4+ populations, respectively. Confocal analysis revealed no difference in the numbers of TRPV1-containing DRG neurons in either group when compared to naïve controls and the contralateral injury (28d) group (Naïve: 33.1 ± 2.1 ; PenCON + contra saph cut (28d): 30.1 ± 1.8 ; PenV1 + contra saph cut (28d): 29.5 ± 2.0) (Fig 17). Total IB4 binding was also unaffected under either condition (Naïve: 34.5 ± 2.5 ; PenCON + contra saph cut (28d): 30.3 ± 2.5 ; PenV1 + contra saph cut (28d): 30.7 ± 2.3). (Fig 17).

Table 8. NB-stained characterized C-fibers in PenV1 and PenCON mice. CPM fibers staining positively for TRPV1 are evident in both PenCON and PenV1 mice at 4-5 weeks following contralateral saphenous nerve axotomy.

	Uninjured		Contra saph cut (4-5 wks)		PenCON + contra saph cut (4-5 wks)		PenV1 + contra saph cut (4-5 wks)	
<i>Neuron Type</i>	IB4+	TRPV1+	IB4+	TRPV1+	IB4+	TRPV1+	IB4+	TRPV1+
CPM	17/26	0/18	11/15	3/15	2/3	1/3	6/8	1/8
CH	0/4	4/4	0/2	2/2	0/1	1/1	0/4	4/4

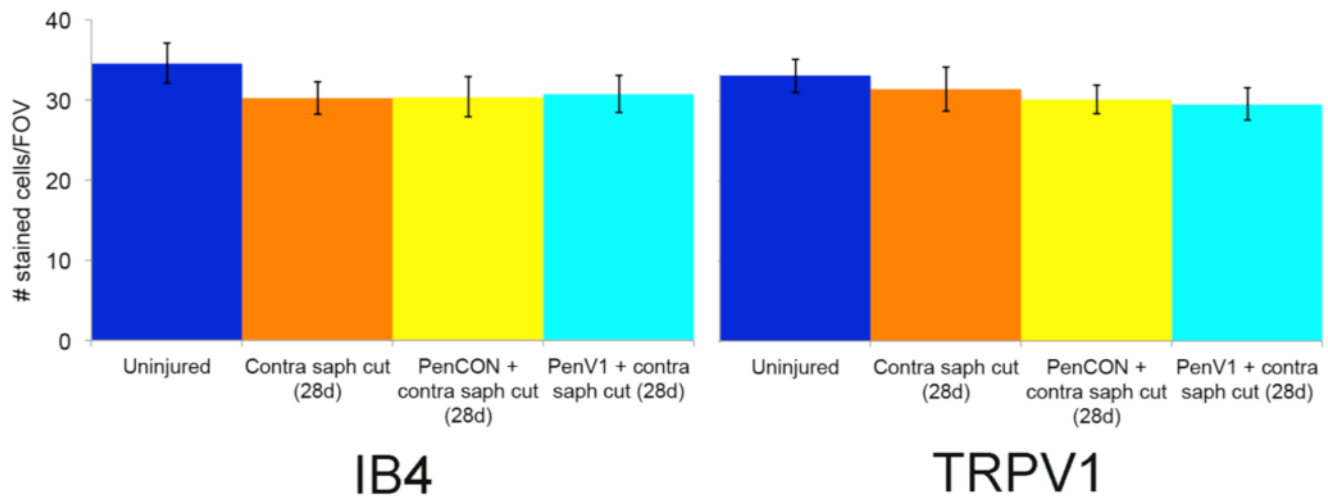


Figure 17. IB4 and TRPV1 staining in L3 DRGs from PenV1 and control mice. There was no change in total TRPV1-staining or IB4-binding in the L3 DRG at 4-5 weeks following contralateral saphenous nerve axotomy in either PenV1 or PenCON mice.

4.0 GENERAL DISCUSSION

The data presented here demonstrates that a unilateral nerve injury resulted in increased mRNA expression of several neurotrophic factors in the uninjured, contralateral skin. The specific neurotrophic factors, including members of the NGF and GDNF families, and the regions of skin affected were largely the same as seen in the nerve injured side (Jankowski et al, 2009a). We found that protein expression of two neurotrophic factors, GDNF and Artemin, was similarly increased in the skin innervated by the contralateral saphenous nerve at 14d following injury.

The increase in GDNF protein was surprising since we did not see a significant mRNA increase until the 21d timepoint. Our data at the 14d timepoint showed a mean 30% increase in GDNF mRNA levels, but the error associated with the measurement was nearly equal in magnitude, suggesting that if there were a small increase in GDNF mRNA levels, it might be very difficult to detect. The result may simply mean that very small upregulation of GDNF mRNA is sufficient to produce robust increases in protein expression. It may also suggest that other mechanisms such as translational regulation and protein degradation may have significantly affected GDNF protein expression. Repeating the experiment at the same timepoint with additional mice might shed some light on how such a dramatic increase in GDNF protein occurs without us observing changes in mRNA. It is also possible that GDNF is not a gene whose mRNA expression correlates well with protein expression. It is established that mRNA

and protein levels do not necessarily correlate. Studies of steady state protein and mRNA levels of several hundred genes in cell culture lines including mouse EML (Tian et al., 2004) and human medulloblastoma cells (Vogel et al., 2010) found that mRNA and protein had a correlation coefficients of only 0.41 and 0.29, respectively. These studies point to the importance of translational regulation, protein stability, and protein degradation in determining absolute protein levels and that mRNA may not always be an appropriate surrogate for protein levels.

The enhanced levels of neurotrophic factors were correlated with increased expression of several associated receptor proteins and ion channels in the DRGs, including several nucleotide receptors, TRP channels, and GFL receptors, similar to changes observed on the ipsilateral side following saphenous nerve axotomy and regeneration (Jankowski et al., 2009a). This increased expression was also correlated with significant changes in the functional response properties of sensory neurons in the intact nerve, many of which mirror changes observed following saphenous nerve regeneration (Jankowski et al., 2009). Specifically, the degree of heat threshold reduction in the CPM population in the 4-5 week group and the apparent recruitment of previously unresponsive TRPV1-positive, CH neurons are nearly identical to that seen in the injured nerve 4-6 weeks following transection. Likewise, we found that some TRPV1+ CH fibers appeared to gain mechanical sensitivity as in the ipsilateral side (Jankowski et al., 2009). None of these changes were evident in the contralateral nerve at 2 weeks, despite upregulation of artemin and several other neurotrophins and neurotrophic factors as early as 7d post-axotomy. Together, these data suggest that prolonged exposure of sensory neurons to upregulated endogenous expression of neurotrophic factors can induce phenotypic changes as well as sensitization in subsets of intact cutaneous nociceptors.

4.1 POTENTIAL MECHANISMS OF SYMMETRICAL CHANGE IN CUTANEOUS EXPRESSION OF TROPHIC FACTORS

Numerous mechanisms have been proposed to explain the emergence of contralateral changes observed in clinical and animal studies, including changes in the peripheral or central nervous systems. Specific peripheral mechanisms suggested include sensitization of contralateral afferents by humoral factors and transmedian sprouting of contralateral neurons near structures that receive bilateral innervation following a unilateral nerve injury (Koltzenburg et al., 1999). Proposed central mechanisms include overlapping central terminals of homologous structures, whereby unilateral injury could then communicate to the contralateral side, and communication in the spinal cord and brainstem via commissural interneurons, through direct neuronal activation or release of specific factors to contralateral neurons (Koltzenburg et al., 1999). Most studies provide evidence in favor of central mechanisms over peripheral ones. For example, heat injury-induced contralateral hyperalgesia was not blocked by ipsilateral C-fiber afferent blockade but was prevented by spinal anesthesia or blockade of sympathetic efferents (Coderre and Melzack, 1987), and neither blockade of C-fiber afferent input nor dorsal rhizotomy affected the mechanical hyperalgesia evident in the contralateral hindlimb following unilateral intramuscular injection of acidic saline (Sluka et al., 2001). Additionally, spinal dorsal horn neurons and thalamic neurons demonstrated increased response to mechanical stimulation of the contralateral hindlimb after unilateral injection of acidic saline (Sluka et al., 2001). As described earlier, another study showed that descending facilitatory pathways from the rostral ventral medulla (RVM) were responsible for initiating and maintaining bilateral hyperalgesia after unilateral intramuscular injection of acidic saline (Tillu et al., 2008). Furthermore, both bilateral

mechanical allodynia resulting from a unilateral sciatic nerve inflammation was reversed by intrathecal delivery of glial metabolic inhibitor and proinflammatory cytokine antagonists specific for interleukin-1, tumor necrosis factor, or interleukin-6, suggesting that glial and proinflammatory cytokine actions in the spinal cord are critical for the maintenance of mirror image pain (Milligan et al., 2003).

In the data presented here, the increased cutaneous expression of neurotrophic factors does not appear to be part of a widespread, systemic upregulation, though there were increases in the expression of Artemin in the hairy thigh skin bilaterally and of GDNF and Neurturin in the ipsilateral thigh. Although we have not identified the source of these changes, it is possible that the incision in the thigh skin during axotomy results in a local increase in cutaneous neurotrophic factor expression and a similar one in the mirror opposite thigh skin. The anatomical specificity of these results suggests that the contralateral effects are not the result of a systemic phenomenon but rather a specific neural mechanism involving the CNS. Additional support for the specificity of contralateral changes following peripheral injury comes from human studies where unilateral capsaicin injections elicited mirror symmetric mechanical hyperalgesia in the several subjects (Shenker et al., 2008). In a second study (Lei et al., 2008), it was shown that intramuscular injections of hypertonic saline into the tibialis anterior muscle elicited symmetric changes in cutaneous blood flow which matched areas of perceived pain. In both of these studies, the changes were observed within an hour of the peripheral injections. Such rapid and precise bilateral communication also suggests that central neural mechanisms are involved.

4.2 EFFECTS OF ENHANCED NEUROTROPHIC FACTOR LEVELS ON C-POLYMODAL FIBERS

The decrease in heat thresholds of the non-peptidergic, IB4-binding CPM population correlated with upregulation of cutaneous GDNF and its receptor GFR α 1 in the DRGs. In addition to GFR α 1, IB4-binding C-fibers also contain the ADP receptor P2Y1, which was also upregulated following contralateral nerve cut. P2Y1 has been shown to be essential for normal thermal sensitivity of cutaneous CPM neurons (Molliver et al., 2011). In addition, we have also recently shown that increased P2Y1 expression following cutaneous inflammation is essential for the observed decrease in CPM heat thresholds (Jankowski et al., 2012). Although the precise intracellular signaling mechanisms downstream of GDNF-GFR α 1 leading to the increased expression of these genes is unknown, the evidence obtained here suggests that the cutaneous upregulation of GDNF, along with increased expression of GFR α 1 and P2Y1 may be involved in the sensitization of intact CPM fibers to heat after contralateral nerve injury.

In addition to heat threshold reduction of CPM fibers, there was a significant decrease in the prevalence of CM fibers. Previously we have shown that the majority of CM fibers are nonpeptidergic, bind IB4, and contain the mas gene related G-protein coupled receptor Mrgprd (Lawson et al., 2008; Rau et al., 2009). Thus, many of these fibers would also contain GFR α 1 and be sensitive to GDNF. This reduction in the prevalence of CM fibers (18% - 2%) is almost identical to the results of our previous study of transgenic mice overexpressing GDNF in the skin, where the prevalence of CM fibers in wildtype mice (19%) was reduced to 3% in transgenic mice (Albers et al., 2006). Taken together, these findings suggest the possibility that

cutaneous GDNF levels and signaling through GFR α 1 could alter phenotype of these cells by regulating the ability of this population of C-fibers to respond to heat.

4.3 EFFECTS OF ENHANCED TROPHIC FACTOR LEVELS ON CUTANEOUS CH-FIBERS

The major functional change observed in the population of peptidergic fibers was a significant increase in the prevalence of CH neurons at 4-5 weeks after contralateral injury. In an earlier study (Christianson et al. 2006), it was shown that the percentage of TRPV1-expressing cutaneous neurons in the L2-3 DRGs is significantly higher (~15-20%) than the percentage of functional TRPV1-expressing CH neurons (~5%). This would suggest that in naïve mice many cutaneous fibers containing TRPV1 do not respond to heat or mechanical stimuli. Increased cutaneous levels of Artemin and possibly NGF may lead to the acquisition of heat sensitivity in these TRPV1-positive neurons.

These results are very similar to that seen following regeneration (Jankowski et al., 2009), and we have shown that this change in phenotype is dependent on increased expression of GFR α 3 (Jankowski et al., 2010). Although GFR α 3 expression is unchanged in the L2-3 DRGs contralateral to the injury, it is possible that the increase in cutaneous Artemin is sufficient to increase GFR α 3 *signaling* in the DRG. It is also possible that GFR α 3 expression is increased, but only in the small subset of CH-fibers and that this change is not detectable at the whole DRG level. This difference between ipsilateral and contralateral expression could be due to the fact

that there is a profound increase in DRG neurons expressing GFR α 3 following injury (Bennett et al., 2000). We also observed a significant upregulation in the expression of the purinergic receptor P2Y2, which has been shown to colocalize with TRPV1 in sensory neurons and is a critical contributor to TRPV1 function and thermal nociception (Malin et al., 2008). Thus, increased levels of P2Y2 may also be involved in the plasticity of TRPV1-expressing afferents.

The other apparent change in this population was the appearance of a population of fibers that stained positively for TRPV1 and responded to both heat and mechanical stimuli. This change in phenotype is similar to that seen following regeneration (Jankowski et al., 2009) and inflammation (Koerber et al., 2010). As in the previous studies, this change was apparently not due to an increase in the total number of TRPV1 expressing neurons, as TRPV1 staining in each of the experimental groups was not different from naïve animals. Additional support for such a phenotypic switch is the lack of IB4 staining in these cells. However, we still do not understand the mechanisms of how this population of peptidergic TRPV1+ neurons may acquire mechanical sensitivity. Further study to better characterize the molecular expression profile and plasticity in these cells will be necessary.

4.4 SKIN INNERVATION PATTERNS ARE UNCHANGED AFTER CONTRALATERAL NERVE INJURY

Having identified that a number of target-derived neurotrophic factors have significantly increased cutaneous expression following contralateral nerve injury and that mice overexpressing

such neurotrophic factors as NGF (Stucky et al., 1999), NT-3 (Albers et al., 1996), and BDNF (LeMaster et al., 1999) have increased skin innervation, we sought to quantify the degree of skin innervation in the hairy hindpaw skin innervated by the intact contralateral saphenous nerve using PGP9.5 as a pan-neuronal marker. Although we found significantly increased mRNA and protein expression of several cutaneous neurotrophic factors, there were no significant differences in innervation density between the experimental groups and naïve mice. These findings would suggest that either the enhanced expression is relatively small when compared to levels seen in the transgenic mice or that ongoing, long-term exposure beyond the timeframe used in this study is essential for changing innervation density.

4.5 INCREASED P2Y1 EXPRESSION IS ESSENTIAL FOR THE CONTRALATERAL INJURY-INDUCED HEAT THRESHOLD REDUCTION IN CPM FIBERS

Utilizing an *in vivo* siRNA-mediated knockdown strategy, we were able to show that in intact saphenous afferents following contralateral nerve injury, PenY1 was able to block the upregulation of P2Y1, both in message and protein. The siRNA-mediated knockdown of P2Y1 upregulation did not have any significant effect on the mRNA expression of the other genes we studied, suggesting its action was P2Y1-specific. PenY1 injection at 14 days and 21 days after contralateral saphenous nerve axotomy completely blocked the CPM heat threshold reduction

normally seen at 4-5 weeks after contralateral injury. There was no effect of knockdown on mean firing rates to thermal stimuli or mechanical thresholds, similar to the effect of PenY1 injection on the CFA-induced CPM heat threshold reduction (Jankowski et al., 2011). These findings suggest that this ADP-binding G-protein-coupled receptor is important for regulating heat signaling in this specific type of cutaneous sensory neuron. The specificity of the knockdown of P2Y1 mRNA and protein levels is an important consideration in the interpretation of these results. One of the inherent risks with using a molecular tool like RNA interference is off-targeting effects of the siRNAs on other genes. While it is not feasible to determine all possible off-target effects, we have determined that there were no effects on the expression other purinergic receptors (e.g. P2X3 and P2Y2) found in CPMs and other sensory neurons. In addition, off-target effects are always a concern when utilizing RNAi techniques, as induction of RNAi in mammalian cells by expression of double-stranded RNA can activate innate antiviral response pathways that may produce off-target gene expression (Alvarez et al., 2006). Previous studies in our lab, however, demonstrated that the Penetratin-linked siRNAs such as those used in the current experiments did not alter mRNA expression of interferon-inducible myxovirus resistance 2 (mx2) or interferon type I-induced and double-stranded RNA-activated kinase (IFN type I), indicating that these siRNAs did not activate the interferon viral response pathway (Jankowski et al., 2009b; Jankowski et al. 2010).

Interestingly, PenY1 injection partially blocked the reduction in overall CM prevalence observed at 4-5 weeks following contralateral saphenous nerve axotomy but did not affect the CH recruitment normally seen. We hypothesized earlier that the reduction in CM prevalence may in part be due to the acquisition of heat sensitivity in a population of CM fibers, so it is possible that increased P2Y1 expression is responsible for this shift in phenotype. Our data in

the present studies and in Jankowski et al. (2012) has suggested that P2Y1 expression plays a critical role in altering the thermal thresholds of CPM fibers. It is possible that some of the C-mechanic fibers in naïve mice are in fact thermally sensitive but have heat thresholds beyond the range of temperatures we apply to the skin in our recording experiments. Such a C-mechano fiber could become thermally sensitive once P2Y1 expression levels reached a certain threshold. If this were true, then the knockdown of P2Y1 might prevent some of this “acquisition” of heat sensitivity, resulting in CM prevalence values closer to naïve mice. Recent work has shown that P2Y1 is physically coupled to PKC α and may exert signaling mechanisms through their interaction (Shah et al., 2012). This might be evidence that P2Y1 is less likely to be a heat transducer and more likely to regulate the expression or function of another heat transducer. Another possibility is that P2Y1 interacts with a mechanosensitive channel at the level of expression or function. TRPM3 is a TRP channel family member that is expressed on small diameter neurons in the DRG and has been implicated in both thermal (Vriens et al., 2011) and mechanical (Inoue et al. 2009) sensation. If TRPM3 were indeed a mechanosensor and its expression was negatively correlated with P2Y1 levels, then P2Y1-targeting siRNA might feasibly increase the prevalence of CM fibers. Complicating this possible interpretation is that there was a nearly identical increase in CM prevalence in control siRNA-injected mice. One possible explanation is that the two siRNA injections at 14d and 21d after contralateral injury resulted in damage to a population of fibers which might have limited or removed their access to elevated neurotrophic factor levels in the skin. This might also explain why the reduction in CM prevalence was not affected in the control siRNA-injected mice in the PenV1 experiments, which had a single injection, rather than two.

It is notable the PenY1 group also showed a small, though not significant, increase in cold threshold, suggesting that P2Y1 expression is broadly involved in setting the thermal thresholds of polymodal C-fibers, not simply the heat thresholds. This would agree with our findings of PenY1-mediated knockdown of P2Y1 in inflamed mice, which resulted in increased heat and *decreased* cold thresholds in CPM fibers (Jankowski et al., 2012). Surprisingly, we saw no change in cold thresholds in mice at 4-5 weeks after contralateral injury, even though P2Y1 was significantly upregulated, suggesting that the effect of P2Y1 in regulating cold sensitivity may have reached its ceiling. Another possible interpretation is that PenY1 injection affected the expression of another gene or set of genes that may be involved in regulating the cold sensitivity of these afferent fibers. These data also seem to support the notion that P2Y1 is not a heat or cold transducer but instead regulates the thermal threshold of a subset of C-fibers. We still do not know what molecule or molecules may be transducing thermal stimuli in these fibers and what the functional interaction with P2Y1 might be. TRPV3 in keratinocytes has been shown to use ATP as a signaling molecule in response to thermal stimulation (Mandadi et al., 2009), so this provides a potential mechanism by which P2Y1 and other nucleotide receptors might modulate the thermal responses of afferent fibers. In these experiments, we found that both TRPV3 and TRPV4 mRNA were upregulated in the skin contralateral to injury, so it is possible that increased TRPV3 and/or TRPV4, together with upregulation of P2Y1, may be responsible for the altered thermal thresholds in CPM fibers.

4.6 INCREASED TRPV1 EXPRESSION IS ESSENTIAL FOR THE RECRUITMENT OF CH NEURONS FOLLOWING CONTRALATERAL NERVE INJURY

Previous work has shown that enhanced artemin levels in the skin can regulate TRPV1 expression in the DRGs (Elitt et al 2006). Others and we have also previously shown that peripheral nerve axotomy increased artemin levels in the skin and increased levels of its receptor GFR α 3 in the appropriate DRGs (Bennett et al 2000; Jankowski et al 2009a; 2010). We recently showed that enhanced signaling through the artemin/GFR α 3 pathway after regeneration caused changes in TRPV1 expression, leading to CH neuron recruitment (Jankowski et al., 2010). In these experiments, we directly tested whether the increase in TRPV1 expression, in the absence of frank injury to the nerve itself, was associated with a specific functional change in the intact afferents. We found that injection of Penetratin-1 conjugated, TRPV1-targeting siRNAs into the saphenous nerve could block the contralateral injury-induced increase in TRPV1 mRNA in the DRGs. Here, we show that TRPV1 knockdown did not alter the contralateral injury-induced decrease in CPM heat threshold, but it was able to block the recruitment of CH neurons observed at 4-5 weeks after injury. These results suggest that transient changes in TRPV1 have a critical role in regulating the properties of distinct populations of sensory neurons. As described above, based on immunohistochemical staining of whole L3 DRGs at 28 days after contralateral injury, there is no change in the total number of IB4-binding or TRPV1-positive cells when compared to naïve mice. The implication of this finding is that the “recruitment” of CH neurons would appear to come from a population of TRPV1-positive cells that do not respond to mechanical or thermal stimuli in naïve animals. We cannot label these cells as functionally silent, as they may

still be chemosensitive, if not mechanically or thermally sensitive. Given that TRPV1 upregulation appears to be essential for CH recruitment, one plausible explanation is that increased TRPV1 expression in some of these neurons reaches a threshold at which point they become responsive to our heat stimulus. Our results strongly suggest that changes in TRPV1 expression are able to regulate the number of functional CH neurons in mice. The ability to correlate TRPV1 expression levels with heat sensitivity at the single cell level may shed light on this possibility.

Among Neurobiotin-stained characterized cells, there were polymodal C-fibers in both the PenV1 and PenCON groups that stained positively for TRPV1, like those seen in the contralateral injury group at 4-5 weeks and the control injection groups. This result is in agreement with our recent findings showed that TRPV1 knockdown did not affect the prevalence of TRPV1+ CPM neurons following nerve injury and regeneration. We still do not know the mechanism by which these TRPV1+ afferents may acquire mechanical sensitivity. Koizumu et al. (2004) showed that mechanical stimulation of cultured keratinocytes evoked release of ATP, a mechanism that might implicate P2Y2, which is highly expressed on TRPV1+ neurons and has been shown to be important for TRPV1 function, in mechanosensation in this peptidergic population. Expression of P2Y2 was significantly increased after contralateral injury, and this increase was not affected by TRPV1 knockdown.

4.7 SUMMARY AND FUTURE DIRECTIONS

The data presented here document mirror symmetric changes in the expression of neurotrophic factors in the skin and specific receptors and channels in the L2-3 DRGs following unilateral nerve injury. These changes in expression are correlated with specific changes in the functional properties of cutaneous fibers in the intact saphenous nerve. These changes differ between the population of GDNF sensitive CPM fibers and the Artemin and NGF sensitive CH fibers. These results show that endogenous upregulation of target-derived neurotrophic factors may lead to pronounced sensory neuron plasticity in intact sensory fibers, not simply those fibers subjected to injury or other pathology. More specifically, we show that transient increases in P2Y1 and TRPV1 expression in the DRG are essential for the observed heat threshold reduction in polymodal C-fibers and the recruitment of CH neurons, respectively. We know that the animals in these experiments are not truly “naïve,” but the fact that intact fibers exposed to increased expression levels of neurotrophic factors show similar increases in the expression of receptor proteins and ion channels in the DRG and exhibit many of the same functional changes as injured neurons suggests that the molecular changes potentiate this phenotypic change. Furthermore, although we have not directly studied pain with these experiments, evidence of neurotrophic factor-mediated sensitization of intact primary afferents contralateral to nerve injury provides an interesting mechanism by which mirror image pain might develop. Additionally, given that clinical nerve trauma does not always involve complete transection of the nerve fascicle, instead leaving intact fibers adjacent to damaged ones, these experiments can help us to understand how the properties of those intact fibers may change following nerve injury and how they might contribute to the development of neuropathic pain.

Of additional interest from a clinical perspective is our finding that some previously silent DRG neurons gain heat sensitivity and that some TRPV1+ CH fibers appear to acquire mechanical sensitivity at 4-5 weeks following contralateral nerve injury, similar to what was reported in regenerated nerves (Jankowski et al., 2009a). Mechanically-insensitive, heat-sensitive fibers have been identified in nonhuman primates (Baumann et al., 1991) and in humans (Schmidt et al., 1995). The results here correlate with findings in patients with erythromelalgia, a neurovascular pain disorder, in which C-fibers with the biophysical properties of mechanically-insensitive afferents appeared to gain mechanical sensitivity (Ørstavik et al., 2003). Patients with diabetic neuropathy were also found to have a dramatic increase in the prevalence of mechanically-insensitive afferents (Ørstavik et al., 2006). The similarity in CH recruitment and acquisition of mechanical sensitivity in CH neurons between the regenerated (Jankowski et al., 2009a) and uninjured contralateral saphenous nerve described here suggests that the target-derived neurotrophic factor signaling we have focused on may be important to the primary afferent plasticity observed in neuropathic pain disorders.

Although many of the functional changes observed in the saphenous nerve at 4-5 weeks after contralateral axotomy mirror those changes seen in the regenerated nerve at a similar time point, these are not identical findings. The intact contralateral saphenous nerve is exposed to increased levels of neurotrophic factors for a much longer period of time within that 4-5 week time frame, although it is possible that the injured nerve is also exposed to increased neurotrophic factor levels as it regenerates through the distal stump. It may also be that although the mRNA changes in neurotrophic factor expression in the contralateral skin appeared similar to that seen in the ipsilateral skin, significant differences in protein levels may exist. Our Western blot analysis of GDNF and Artemin protein in the skin innervated by the intact

saphenous nerve showed a similar increase relative to naive mice to what was observed on the injured side, but this analysis was done at 14d after injury, and the symmetry may not hold at later time points. The similarities and differences between the injured and contralateral sides may provide important insight into the differential behavior of neurotrophic factor expression on injured and intact afferents and establish which plastic changes are unique to damaged nerves and which can also occur in the context of healthy, undamaged nerves. Further investigation will be needed to elucidate the specific signaling mechanisms responsible for the changes (or lack of change) observed.

Given that exogenous administration of GFLs such as GDNF and Artemin have been shown to sensitize cultured DRG neurons *in vitro* and produce acute thermal hypersensitivity *in vivo* (Malin et al., 2006), the lack of any apparent functional plasticity at the 14d timepoint, where Artemin and GDNF protein were significantly increased, is somewhat surprising. In a CFA model of inflammation, there was not only increased expression of NGF and Artemin in the skin, but both TRPV1 and P2Y1 mRNA were acutely upregulated in the DRGs as well (Malin et al., 2006). One potential explanation is that protein levels of Artemin and NGF are significantly lower in the contralateral injury model used here relative to acute inflammation. Interestingly, combined injection of NGF and artemin into the skin resulted in significant thermal hyperalgesia lasting 6 days, several days more than what was seen when any individual factor was injected (Malin et al. 2006). Also, NGF/artemin-induced hyperalgesia was significantly greater than that induced by artemin or NGF alone at 4 h after injection. These data point to a potentially synergistic effect of trophic factor expression, possibly via potentiation of DRG targets such as TRPV1, in modulating the sensitivity of nociceptors *in vivo*. Here, we observed cutaneous increases in NGF, NT-4/5, Artemin, and GDNF, suggesting that the effects of these factors way

work synergistically to modulate nociceptor function chronically. The lack of acute primary afferent plasticity here may indicate that the levels of trophic factor expression in the contralateral skin, while significantly elevated, are smaller than the changes seen in injured or inflamed nerves but that increased trophic factors can still alter nociceptor function through the long-term modulation of receptor proteins and ion channel expression in the DRG. As discussed earlier, single cell analysis of characterized afferents is a powerful tool that may allow us to directly correlate mRNA expression levels in individual sensory neurons with their response properties. Such analysis would address the difficulty of detecting small changes in expression in a small population of cells and could reveal the trophic factor sensitivity of neurons that were or were not sensitized. This avenue of investigation might also provide novel targets in the study of sensory neuron plasticity. In the experiments here, for example, we might be able to characterize a cell's response to thermal stimuli and then directly correlate the heat threshold or firing rate to the expression level of P2Y1 or TRPV1 in that neuron.

Having shown previously (Jankowski et al., 2010, 2011, 2012) and here that siRNA injection can selectively block upregulation of a target gene in a localized fashion and affect nociceptor function, a natural question is what implications these studies may have for the development of therapeutic agents for persistent pain. Though none has looked at trophic factor expression, several studies using experimental animals have shown that RNAi can be used to prevent or attenuate the development of neuropathic (Dorn et al, 2004; Dong et al., 2007; Wu et al., 2010; Le et al., 2011; Sun et al., 2012; Tsantoulas et al., 2012) and visceral pain (Christoph et al., 2006). Trophic factors antagonists such as anti-NGF antibodies and anti-GDNF antibodies have both been shown to diminish inflammatory hyperalgesia and the expression of TRPV1 in the DRG (Amaya et al., 2004). More recently, an anti-NGF monoclonal antibody (Tanezumab)

was shown in clinical trials to have a good treatment profile for osteoarthritic pain (Schnitzer et al., 2011; Nickel et al., 2012) and low back pain (Katz et al., 2011), but one trial was eventually discontinued due to accelerated joint degeneration in enrolled patients. One of the potential benefits of using RNAi techniques such as the siRNA-based method used here is that it allows one to specifically target particular molecules in identified afferents without administration of systemic drugs and without inducing sub-baseline expression levels or neuronal activity.

There are a number of obvious concerns about any approach using RNAi, however. As discussed earlier, induction of RNAi in mammalian cells by expression of double-stranded RNA can activate innate antiviral response pathways that can produce off-target gene expression (Alvarez et al., 2006), even though the specific technique described here was not correlated with any significant activation of the interferon viral response pathway (Jankowski et al., 2009b; Jankowski et al. 2010). There is also potential concern about the transient nature of the siRNA-mediated knockdown. Indeed we have shown that siRNA produces significant knockdown of P2Y1 and TRPV1 lasting 1-2 weeks after the initial injection, but the signaling mechanisms that are responsible for increasing P2Y1 and TRPV1, for example, may simply produce the same increase in expression once the siRNA's effect passes. Indeed our previous study showed that GFR α 3-targeting siRNA blocked CH recruitment after regeneration and TRPV1 upregulation at 4-6 weeks after nerve injury, but by 10 weeks, significant CH recruitment was evident, and TRPV1 expression had rebounded from its baseline level at 4-6 weeks (Jankowski et al., 2010). Clearly, engagement of the RNAi pathway has potential in the development of treatments for persistent pain, but identification of multiple molecular targets and engineering a means to deliver enough quantity of the siRNAs into the target tissue to produce significant and lasting knockdown without requiring repeated injections are substantial hurdles. In the meantime, the

siRNA-mediated strategy we use here provides a valuable tool for investigating the role of transient changes in expression of specific genes in neuronal plasticity.

APPENDIX A

REAL-TIME RT-PCR PRIMER SEQUENCES

Below are forward and reverse primers that were used for real-time RT-PCR experiments.

From Elitt et al. (2006)

GFR α 3: forward, 5'-CTTGGTGACTACGAGTTGGATGTC-3'; reverse, 5'-AGATTCATTTTCCAGGGTTTGC-3'

TrkA: forward, 5'-AGAGTGGCCTCCGCTTTGT-3'; reverse, 5'-CGCATTGGAGGACAGATTCA-3'

TRPV1: forward, 5'-TTCCTGCAGAAGAGCAAGAAGC-3'; reverse, 5'-CCCATTGTGCAGATTGAGCAT-3'

TRPV2: forward, 5'-CCAGCCATTCCCTCATCAAAA-3'; reverse, 5'-AAGTACCACAGCTGGCCCAGTA-3'

TRPV3: forward, 5'-TGAAAGAAGGCATTGCCATTT-3'; reverse, 5'-GAAACCAGGCATCTGACAGGAT-3'

TRPV4: forward, 5'-TGGATTCCTTGTTTCGACTACGG-3'; reverse, 5'-CACAATGTCAAAGAGGATGGGC-3'

TRPA1: forward, 5'-GCAGGTGGAACCTTCATACCAACT-3'; reverse, 5'-CACTTTGCGTAAGTACCAGAGTGG-3'

ASIC1: forward, 5'-CTGGACTTCCTAGTGGAGAA-3'; reverse, 5'-CCAGCACCAAGAATATTCTCC-3'

ASIC2a: forward, 5'-ATGGACCTCAAGGAGAGCCCCAG-3'; reverse, 5'-AAGTCTTGATGCCCACACTCCTGC-3'

ASIC2b: forward, 5'-CGCACAACTTCTCCTCAGTGTTTAC-3'; reverse, 5'-TTGGATGAAAGGTGGCTCAGAC-3'

ASIC3: forward, 5'-ATGAAACCTCCCTCAGGACTGG-3'; reverse, 5'-AACTCCCCATAGTAGCGAACCC-3'

GAPDH: forward, 5'-ATGTGTCCGTCGTGGATCTGA-3'; reverse, 5'-ATGCCTGCTTCACCACCTTCTT-3'

From Jankowski et al. (2009):

Nerve Growth Factor (NGF): forward, 5'-ACA CTC TGA TCA CTG CGT TTT TG-3'; reverse, 5'-CCT TCT GGG ACA TTG CTA TCT GT-3'

BDNF: forward, 5'-CCA TAA GGA CGC GGA CTT GT-3'; reverse, 5'-AGGAGGCTCCAAAGGCACTT-3'

NT-3: forward, 5'-CCT GTG GGT GAC CGA CAA G-3'; reverse, 5'-GAT CTC CCC CAG CAC TGT GA-3'

NT-4/5: forward, 5'-GGA GGC ACT GGC TCT CAG AAT-3'; reverse, 5'-CCC TGG GAG TCT GCA GTC AA-3'

Glial Cell Line Derived Neurotrophic Factor (GDNF): forward, 5'-AGC TGC CAG CCC AGA GAA TT-3'; reverse, 5'-GCA CCC CCG ATT TTT GC-3'

Neurturin: forward, 5'-TGA GGA CGA GGT GTC CTT CCT-3'; reverse, 5'-AGC TCT TGC AGC GTG TGG TA-3'

Artemin: forward, 5'-GGC CAA CCC TAG CTG TTC T-3'; reverse, 5'-TGG GTC CAG GGA AGC TT-3'

p75: forward, 5'-GGG TGA TGG CAA CCT CTA CAG T-3'; reverse, 5'-GTG TCA CCA TTG AGC AGC TTC T-3'

trkB: forward, 5'-GCT GAT GGC AGA GGG TAA CC-3'; reverse, 5'-GCG ATT TGC TGA GCG ATG T-3'

trkC: forward, 5'-GCT TTC CAA CAC GGA GGT CAT-3'; reverse, 5'-GCA GAC TCT GGG TCT CTC CAA-3'

GFR α 1: forward, 5'-GTG TGC AGA TGC TGT GGA CTA G-3'; reverse, 5'-TTC AGT GCT TCA CAC GCA CTT G-3'

GFR α 2: forward, 5'-TGA CGG AGG GTG AGG AGT TCT-3'; reverse, 5'-GAG AGG CGG GAG GTC ACA G-3'

P2X3: forward, 5'-TGG AGA ATG GCA GCG AGT A-3'; reverse, 5'-ACC AGC ACA TCA AAG CGG A-3'

P2Y1: forward, 5'-GGC TAT CTG GAT GTT CGT TTT CC-3'; reverse, 5'-CCA GAG CCA AAT TGA ACA TGT ACA-3'

P2Y2: forward, 5'-TTG GCA GGG GCT CAG GA-3'; reverse, 5'-GCA TAG GAG TCG GGT GCA-3'

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